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A Physiological Study on the Amino Acid Requirements of Sphaerophorus necrophorus

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A PHYSIOLOGICAL STUDY ON THE AMINO ACID REQUIREMENTS OF *SPHAEROPHORUS NECROPHORUS*

BY

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A PHYSIOLOGICAL STUDY ON THE ANIMO ACID REQUIREMENTS

OF SPHAEROPHORUS NECROPHORUS

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Investigation into the growth requirements of Sphaerophorus necrophorus (Flugge, 1886) Prevot, 1938 presents a difficult area for study. Only a few such investigations have been attempted in the past for this highly pleomorphic, gram negative, thread-like micro-organism. At present, there is a great deal of confusion concerning its growth characteristics. Most of this confusion appears to be the result of a deficiency in the basic knowledge about the growth habits peculiar to the organism. Moreover, the utilization of complex media containing unknown nutrients in the past has served to further confuse information about the organism's growth characteristics. The development of a chemically defined medium will certainly play an important role in obtaining consistent growth characteristics which are more typical of the organism. Such a medium could serve to clarify the descriptions of the organisms' cultural and morphological characteristics. Experimental inconsistencies resulting from the organisms' growth requirements could also be corrected (29, 13, 57).

The importance of this organism has been centered upon its role as a pathogen (15). As a cause of necrobacillosis diseases of domestic and wild animals, and numerous infections in man, this organism is characterized by its ability to produce necrotic tissue and lesions on various organs in warm blooded animals (34, 47). It is especially characterized by its ability to form bovine liver abscesses resulting in a three year retail loss for the period of 1957-59 of eight million dollars for twenty four million pounds of condemned bovine

livers (36, 59). Jensen et al. (35) have stressed the relationship between liver abscesses in cattle and the feeding of rough or fattening rations to beef cattle. This relationship is further emphasized by a study on the incidence of liver abscesses in cattle where one group of steers was fed a high level of fat. This study has recently been completed by the Animal Husbandry department at South Dakota State College (67). One lot of 196 steers had 35 livers condemned for abscesses and a second lot of 275 steers had 30 livers condemned. This represents 18.4 and 10.9 percent condemnation respectively. In the lot of 196 steers, 99 were fed a high concentration (three percent by weight in the feed) of prime yellow grease. The importance of fat in the diet is shown by the fact that out of 99 steers receiving the fat 23 developed abscesses compared to 12 from a lot of 97 receiving no fat. The former represents 23.2 percent abscessed livers while the latter represents 12.4 percent abscessed livers.

Sphaerophorus necrophorus is best identified by the presence of pleomorphic, swelling producing filaments in bovine liver abscesses; a characteristic fetid odor (of acid sharpness), the production of large amounts of gas, the production of nodules in an agar medium, and its anaerobic requirement serve to further identify the organism (11, 52, 50).

The difficulties existing in the classification of this organism are clearly indicated in the bacteriological classification manuals of Bergey (United States) and Prevot (France) (11, 52). Mansson, Labelle and Thjotta (Norway) (40) have published several papers suggesting that the genus name Necrobacterium Jonsen and Thjotta would better describe the organism than does the genus name Sphaerophorus Prevot. Each of

these schemes for classifying the organism demonstrates the inconsistancies inherent in the employment of the usual characteristics. These usual characteristics include morphology, biochemical reactions, culture media employed, pathogenicity, and the organisms habitat or isolation source. In recent years, other characteristics have been described by the employment of new and novel methods. Among these new methods are included the bacteriostatic effect of dyes, hemagglutination, and the type of fermentation reaction given by the organism (3, 4, 61, 5, 31, 32).

The continued use of complex, chemically undefinable, culture media in these studies will serve to continue the confusion concerning the identifying characteristics. Unless a simple medium containing chemically identifiable nutrients can be developed, this confusion will most certainly persist. This simple, chemically defined medium could be used in standardizing the procedures involved for observing the morphological and cultural characteristics. These studies would profit by the minimization of interferring factors offered by a chemically defined medium (29).

Research on synthetic or chemically defined media for this organism began with the study by West and Lewis (66). They considered the nutritional requirements of twenty nine strains of gram negative, non-spore-forming anaerobic bacteria from the intestinal tract. Their main objective was to find a growth factor or nutrient that would serve as a growth stimulator and thereby improve on the otherwise uncertain and erratic growth shown by these organisms on culture media. Seventeen amino acids, in addition to the mineral salts, glucose, glycerol, and

sodium lactate were the components of the basal medium employed. The results of this investigation ended in failure of the synthetic medium to support growth. Grant (29) followed this work with another study on growth stimulatory nutrients. His duplication of West and Lewis synthetic medium also met with the failure of the medium to support the growth of several strains of S. necrophorus. By fractionating casitone, he demonstrated that it possibly contains a growth factor that he was unable to identify. Guillaume et al. (32), on the other hand, have been successful in cultivating a Spherophorus freundi (Hauduroy et al. 1937) Prevot strain on a synthetic medium composed of thirteen amino acids, glucose, glycerol, sodium acetate, and vitamins of the B complex.

Grant's (29) fractionation of Difco fluid thioglycollate medium (22) and identification of the constituents essential for growth serves as the basis for the present study. The investigation of the amino acid requirements by S. necrophorus was divided into three parts. The first involves replacing the casitone portion of fluid thioglycollate medium with simpler caesin break-down products until finally the amino acid requirements were determined. Further modification of Grant's thioglycollate basal medium encompasses the second part while the third deals with the individual amino acids required by the organism.

In order to evaluate the effect of each nutrient exposed to the action of S. necrophorus, optical density readings were used. These readings are inversely proportional to the percent transmission of light through the culture medium (2, 49, 57). A Coleman photoelectric colorimeter with a 590 Mu filter served as a means for making a growth index measure of the growth responses demonstrated for each nutrient tested.

One isolant, S7, was singled out for its typical, vigorous, uncontaminated growth characteristics for consistent use throughout the present study. Other isolants are used to validate growth of the S7 isolant on certain test media at various places in the amino acid study.

Definition of Terms Used

Isolant -- refers to organisms obtained from different sources or abscesses. Their identities are established only by their morphological and cultural characteristics and not by more involved characteristics such as biochemical reactions.

Growth factors -- consist of vitamins, amino acid-like compounds and other chemical structures which serve to further stimulate growth. These factors are usually required in minute amounts and may be quite essential in the continuance of growth by an organism.

Basal medium -- used for the study of the growth responses caused by different nutrients. This medium is characterized by its simplicity. It is composed of only the nutrients necessary for growth. This term has been applied to two types of culture media.

Incomplete -- one component has been left out, usually the component most essential for growth. Comparatively little growth occurs on this type of basal medium. This medium is used to measure the growth responses of various nutrients.

Complete -- all of the essential components are present. Optimum or maximum growth occurs with this medium, which can serve as a control in comparing the growth response resulting from a nutrient which has been added to the incomplete medium.

Chemically defined or synthetic medium -- consists of a nitrogen source and a carbon source, an energy source, and minerals all of which are chemically known and essential for growth. Growth factors, accessories or vitamins of known chemical structure may also be included to increase growth or improve on the continuance of growth.

Complex or chemically undefinable organic medium -- contains growth nutrients of a complexity that have not been analyzed as to their qualitative and quantative chemical nature. Meat extract and yeast extract are representatives of such a group of chemically undefined nutrients.

Significance of Optical Density Readings

O.D. of 0.0 to 0.05 -- no growth to only slight growth

O.D. of 0.05 to 0.1 -- slight to fair growth

O.D. of 0.1 to 0.5 -- fair to good growth

O.D. of 0.5 to 1.0 -- good to excellent growth

From an optical density reading of 0.45 and on up the growth is so heavy that the culture takes on a viscous appearance. At 0.65 to 1.0 optical density, the culture broth is almost gel-like in nature and requires more time in swirling to fully distribute the cells more evenly.

REVIEW OF LITERATURE

Sphaerophorus necrophorus (Flugge, 1886) Prevot, 1938 is a member of a rather unique group of bacteria. Almost a mold-like organism, this highly pleomorphic thread-like micro-organism is representative of a group of bacteria characterized by being anaerobic, gram negative, and non-sporeforming, with a close relationship exhibited between some of the members. This group of anaerobic bacteria received very little attention for a number of years because it was believed that they played only a minor role in pathological conditions since they were rarely found. These organisms were generally found only when they produced an abscess or invaded the blood stream. Later investigations with better culturing techniques and improved media showed that they were present in large numbers as part of the normal flora of the mucous membranes of warm blooded animals. The necrotic infections produced in man and animals is the distinctive feature of the type species S. necrophorus (11, 15).

Bergey's Manual of Determinative Bacteriology (11) describes the organism as being extremely pleomorphic, especially in cultures. Rod forms measure 0.5 to 1.5 by 1.5 to 3.0 microns in pathological processes. The filamentous forms may measure 80 to 100 microns in length. Lahelle and Thjotta (40) measured a filament which was 700 microns in length. They also reported that the length of the filaments may vary and usually possess rounded ends, although the ends may also be truncate or pointed. Within these filaments they observed darker granules which were also present in the rod forms. The thread-like elements appeared to have

spool-like protuberances on the bacterial body or even round, large isolated yeast-like bodies (40). Orcutt (50) observed the long rods and filaments in young cultures whereas older cultures exhibited shorter rods of varying length. Coccoid forms and beaded rod forms, which are characteristic when stained with carbol fuchsin, with spore-like areas were also observed. Bergey's Manual (11) further states that branching forms have been reported by some authors and Schmorl regards shorter forms as being motile; whereas, Lahelle and other recent investigators report the species to be non-motile and non-flagellated. Prevot (52) defines the genus as being pleomorphic with dipolar staining in exudates. In culture, the forms will vary from filaments to rods to ovoid forms. There is a constant presence of spheroids of varying sizes. Occasionally the large body, liberated on setting, contains chromatin. The filaments contain metachromatic granules. Canada (12) and Smith (56) report the presence of septa or granules within the organism.

According to Bergey's Manual (11), S. necrophorus is an anaerobic organism with optimum growth between 30°C and 40°C. The optimum pH for growth is between 7.5 and 7.8 although growth occurs within a pH range of 6.0 to 8.5 (47).

Law (41) mentioned a report by Klienberger-Nobel (39) on an L-phase of growth for S. necrophorus. The significance of such a phase of growth is the sexual reproduction characteristic involved whereby bacteria are transformed by a process of fusion and regeneration. Dienes et al. (18, 19, 20, 21) and Prevot and Raynaud (53) believe that the presence of naturally or voluntarily produced spheroids by S. funduliformis (Halle, 1898) Prevot (synonymous to S. necrophorus) strains

is responsible for this type of reproduction. Both authors have reported on the presence of nucleus-like chromatic bodies within the swollen forms which may break up into granules and either produce a peculiar pleomorphic growth or reproduce an ordinary bacteria cell growth. Prevot and Raynaud (53) suggest that the presence of spheroids signifies that two types of reproduction can occur in the Sphaerophorus group of anaerobes. The first involves a simple type, that of binary fission. The second type is a complex, sexual form of conjugation, forming spheroids. This form involves granules arranging to "germinate" as a bacillary form. Some authors, according to Bergey's Manual (11), have reported that branching can occur. This branching suggests a third type of reproduction for S. necrophorus by means of a specialized form of budding (23).

Sphaerophorus necrophorus has been blamed for everything from ingrown toenails to falling hair (8). Canada (12) lists a wide range of hosts including man, wild and domestic animals, reptiles, chickens, and a species of French termites. The organism is believed to be, at present, the normal inhabitant of mucous membranes of the body such as is found in the upper respiratory tract, colon, and the genital tract (11, 56). It has generally been found only when it produced abscesses or invaded the blood stream. Laboratory animals susceptable to infection by this organism include rabbits, white mice and guinea pigs (11). On the other hand, white rats are resistant and guinea pigs have been, in the past, listed as resistant if the diet is not deficient in essential nutrients or if vitamin C is present in the diet (56, 44).

Necrobacillosis serves as a collective name for diseases in which S. necrophorus appears as the predominating organism (34). Fitch (25)

in 1919 summarized the importance that some of the early workers demonstrated for the organism involved in the necrobacillosis diseases of animals. Flugge in 1886 described an organism causing calf diphtheria that he named Bacillus diphtheriae vitulorum. This description so closely matched that for Bacillus necrophorus that thereafter the type species name was known as B. necrophorus Flugge. Previous to 1886, Koch observed the organism in sheep pox in 1881. Three years later Loeffler described the etiological difference which existed between diphtheria in man and in cattle and showed that the action of two different micro-organisms was involved. Schutz and Theobald Smith observed the organism to be associated with the inflammation of the intestinal mucosa in swine, and with hog cholera. Bang in 1891 established the identity of the organisms seen by the previous workers and gave the name Nekrosebazillus to the organism (25).

According to Flint and Jensen (26), S. necrophorus was first isolated from "foot rot" in sheep and reindeer in 1905. Later reports in 1922 and 1947 described foot rot or "fouls" in cattle from which S. necrophorus was isolated (46). In 1940 a virus was thought to be involved, with S. necrophorus entering as a secondary invader. Beveridge (56, 64, 46) in 1941 demonstrated that the primary agent in sheep foot rot, both in Australia and in the United States, was the organism he named Fusiformis nodosus (Hoelling) Beveridge. This organism was later designated as Bacteroides nodosus (Castellani and Chalmers) Beveridge. Furthermore, he contended that the organism which he called Spirochaeta penortha (Nemen muda) Beveridge acted as an accessory agent. S. necrophorus strains predominating in foot rot are apparently not found among the normal flora of the gastro-intestinal tract and do not grow nor long

survive in wet or dry soil. Apparently sheep often harbor small, inconspicuous lesions in which the organism will remain viable for months. S. necrophorus cannot by itself experimently produce the disease, whereas B. nodosus produces a less severe form of foot rot when introduced into the foot of an animal. The severity of the B. nodosus infection increases when the spirocete is also injected into the foot. Hagan and Bruner (34) refer to the organism as Actinomyces nodosus (Harz) Beveridge. The two predominating organisms according to the observations of Hagan and Bruner have been the spirocete and a motile, fusiform bacillus whose function is unknown. B. nodosus and Sp. penortha do not appear to infect laboratory animals and will only cause lesions in the scarified skin around the margins of the claws of sheep.

Madin (45) reported that 89.2 percent of beef liver abscesses contained S. necrophorus. Newsom (48) mentions that hemolytic diphtheroids and a coccoid organism are also found in the liver abscesses. Jensen, Connell, and Deem (35) found that liver abscesses can also be caused by the invasion of different types of pyogenic bacteria with the Streptococcus and Staphylococcus types the most common of these invaders. The size of the beef liver abscesses may vary in diameter from a very small abscess (several millimeters) to a very large abscess (several centimeters)(56). Frederick (28) describes the liver lesions as being spherical or irregular, rather sharply defined, nodular areas, yellowish or grayish-yellow and of a solid or compact, brittle consistency. These nodular areas vary from five tenths to two inches in diameter. The lesions will occasionally fuse into more extensive lesions. According to Frederick, these lesions are characteristic enough to aid in diagnosis of a necro-

bacillosis type of disease.

Madin (45) quoted a classical description given by Bang for describing the lesions of bovine livers.

> The disease is usually found in healthy appearing animals at slaughter. The liver is more or less enlarged, and contains as a rule a greater or lesser number of walnut to egg-sized abscesses, which lie partially in the depths of the organ, and partially on the surface prominences. The abscesses are surrounded by a thick (externally fibrous, internally soft) capsule, and contains a thick tenacious pus.
>
> Embedded in this, one finds on closer examination solid clumps similar in size to the necrotic areas previously described. If one cuts through such a clump one sees that it consists of homogenous dry, grey, necrotic tissue. On the outside of these clumps, and in the pus I have found the necrosis bacillus; through inoculation the identity of the organism occuring in liver necrosis was established.

Jensen and Flint (36) used intraportal inoculation with viable pure cultures of S. necrophorus and were able to produce liver abscesses. They observed that the abscess formation began eight days after inoculation and by thirty days maximum size was obtained; moreover, the central necrotic liver tissue persisted for as long as one hundred days. Canada (12) gave a description of two other conditions of the liver which occur along with the abscesses, telangiectasis and "sawdust liver." Jensen, Frey, Cross, and Connell (37) suggested that telangiectasis predisposes liver tissue to abscesses much as rumen ulcers preceed abscesses (35, 55). This hemorrhagic infarction consisting of a single or multiple dark red foci is transformed to the grey colored foci of the "sawdust liver" by the hemolysis of erythrocytes and the infiltration of leucocytes. The dead tissue present in these conditions constitute a favorable medium for S. necrophorus. Hagan and Bruner (34) state, "The evidence indicates that this organism has little or no ability to invade normal mucous

membranes of the skin but that it frequently thrives in wounds of the surfaces produced by mechanical injury or bacterial action." They further point out that liver abscesses may be an exception because the organism is usually present in pure culture and hence it can not be considered purely a saprophytic type.

Isolation from the beef liver abscesses has been accomplished in the past by searing the surface with a spatula, making an incision with a sterile scapel heated red hot, and withdrawing samples of pus on a sterile inoculating loop (29, 36, 50, 48). Both Canada (12) and Law (42) used hyperdermic syringes and eighteen-gauge needles. The abscess surfaces were sterilized with alcohol and a sample of the pus was drawn into the syringe which contained one tenth to two tenths milliliters of thioglycollate broth. A small sample of pus was thereby collected and transferred to the thioglycollate medium.

Hagan and Bruner (34) and Canada (12) report the production of two toxins by the organism; a soluble exotoxin and a soluble heat stable, chemical resistant endotoxin. When the endotoxin was injected into the interperitoneal cavity of laboratory mice, according to Hagan and Bruner, it was found to be toxic to the mice. The exotoxin appeared to be of low order toxicity towards the mice when injected into the intraperitoneal cavity. Merchant (47) points out that an edema occurs when the exotoxin is injected intradermally into rabbits. The production of a necrotizing endotoxin by the organism contributes to the organisms ability to produce lesions in animal tissue.

Although the organism is considered a strict anaerobe, this characteristic is at times questionable. Hagan (33) suggests that the

formation of hydrogen peroxide, which accumulates in from four to six hours, has a definite injurious effect as was demonstrated by the exposure of cultures to air in shallow layers. Dack (15) used desiccator jars, an oil vacuum pump, and carbon-dioxide flushing to grow the organism out on streaked blood agar plates. Grant (29) modified this procedure by using alkaline pyrogallol to absorb any residual oxygen remaining in the jar after evacuation, and sodium carbonate plus hydrochloric acid to increase the carbon dioxide tension within the jar. Lahelle and Thjotta ignited an atmosphere of hydrogen gas within an air-tight container (called a "hydrogen bomb") in order to establish an anaerobic environment suitable for the growth of the organism on agar plates. Guillaume et al. (32) and Beerens (5) sealed their culture tubes under vacuum when studying the breakdown of threonine and serine in a medium containing veal infusion. Hagan (33) suggested the use of meat fragments in culture fluid because these fragments naturally contain heat resisting substances in small amounts acting as peroxidases. Valley (65) suggested the use of cysteine since this appears to serve as an excellent reducing agent in the medium. Grant (29), Law (41), and Canada (12) used fluid thioglycollate medium (thioglycollic acid and cystine) for maintaining anaerobic conditions. Beveridge (9), on the other hand, could maintain viable surface colonies on agar plates or viable growth in broth exposed to air for as long as fourteen days after the initial growth under anaerobic conditions had started. Both Grant (29), and Beerens et al. (5) were able to spin the cells down and wash them twice with sterile buffered saline without any loss of virulence.

Ever since Flugge described an organism causing calf diphtheria

in 1886, the classification of the type species, S. necrophorus, has been a problem for systematic bacteriologists (34, 25, 47). Hagan and Bruner (34) wrote, "Our knowledge of the non-spore-bearing obligate anaerobes is fragmentary, and their classification is confused and certain to be changed as more information about them is acquired." Although there are a number of species listed under the genus Sphaerophorus, the only one of significance to animal disease is S. necrophorus (47).

Canada (12) lists 14 synonyms taken from three reference sources. Ten additional synonyms are also present and are listed in Bergey's Manual (11) and in Prevot's Manual (52). They include, Bacterium funduliforme, Dack; Bacillus filiformis, Shutz only; Nekrosebacillen, Bang, 1890; Actinomyces cuniculi, Gasperini and Mittheil, 1894; Oospor diphtheriae vitulorum, Lehmann and Neumann, 1896; Streptothrix necrophora, Kitt and Bakterienkunde, 1899; Corynebacterium necrophorum, Lehmann and Neumann, 1907; Bacillus necroseos, Salomonsen; Cladothrix cuniculi, Maci and Traite, 1913; and Bacillus der Kalberdiphtherie, Loeffler and Mitteil, 1884.

At present, classification of the organism remains unsolved. Thjotta, Breed and Prevot, although members of the International Bacteriological Nomenclature Committee, disagree on the classification of this organism as shown by the following schemes of nomenclature used by each.

The first classification scheme is according to the seventh edition of Bergey's Manual of Determinative Bacteriology, 1957 (11).

Division I Protophyta Sach, 1874, emend. Krassilnikor, 1949.
Class II Schizomycetes von Naegeli, 1857.
Order IV Eubacteriales Buchanan, 1917, order of true bacteria.
Family VI Bacteroidaceae Breed, Murry, and Smith, fam. nov., pleomorphic rods.

Genus IV Sphaerophorus Prevot, 1938, 18 species, pleomorphic, strict anaerobes, produces spheroids and filaments.
Genus I Bacteroides Castellani and Chalmers, 1919, rounded ends, simple, strict anaerobes.
Genus II Fusobacterium Knorr, 1922, pointed ends, simple, strict anaerobes.
Genus III Dialister Bergey et al., 1923, minute, associated with influenza, but are smaller and filterable rods, simple, strict anaerobes.
Genus V Streptobacillus Levaditi, Nicolau and Poincloux, 1925, facultative anaerobes, pleomorphic, L-type growth.

Prevot's Manual, Classification et Determination des Bacteries Anaerobies, third edition, 1957 (52) is used as the second classification scheme.

Embranchement Schizomycetes
Sous-embranchements Mycobacteria, Bacteria presently related together with the lower fungii.
Class Actinomycetales Prevot, 1938, pseudomycelium.
Ordres Actinobacteriales Prevot, 1938, not acid fast.
Familles Spherophoraceae Prevot, 1938, gram negative, anaerobic, pleomorphic.
Genre I Spherophorus Prevot, spheroids, dipolar rods, metachromatic filaments, Type species S. necrophorus, S. funduliformis.
Genre II Spherocillus Prevot, motile and flagellated. S. bullosus, motile.
Genre III Fusiformis Hoelling, spindle shaped, F. fusiformis Vincent.
Genre IV Fusocillus Prevot, motile, spindle shaped.
Genre V Leptotrichia Trevisan, tapering long rods or filaments.

The first edition of Bergey's Manual, 1923 (6), listed the species name necrophorus as species number 10 of Genus II, Actinomyces Harz belonging to Family I, Actinomycetaceae Buchanan of Order II, Actinomycetales Buchanan. At that time it was known only to cause calf diphtheria and multiple sclerotic abscesses in cattle, and that it was transmissable to mice and rabbits.

By the time that the fifth edition came out in 1939 (7), the classification was changed and the species name necrophorus was placed in Appendix I of the Genus VIII Bacteroides Castellani and Chalmers. This included, "... Bacteroides-like organisms fairly well described whose taxonomic relationships are not clear." Group number 4 of this appendix consisted of, "... those organisms that Prevot places in his genus Spherophorus, ... 1. Actinomyces necrophorus." The Order had been changed to Order I Eubacteriales Buchanan and this Order included Family VI Bacteriaceae Cohn. The description now included additional pathogenic processes such as gangrenous dermatitis in horses and mules, multiple necrotic foci in liver of cattle and hogs, and one case of human infection.

Nine years later, in 1948, the sixth edition (10) further modified the classification to include a Sub-Order and a Tribe, and to remove the species from the Bacteroides appendix. Under the Order I of Eubacteriales Buchanan was listed the Suborder I of Eubacteriineae Breed, Murray and Hitchens. Family XI of Parvobacteriaceae Rahn, under which was included Tribe III Bacteroideae Rahn, was used to designate the anaerobic organisms. Spherophorus necrophorus (Flugge) Prevot was listed under the Appendix II of the Genus I Bacteroides Castellani and Chalmers as a member of the Family Spherophoraceae Prevot. A description of the organism was included, "Because of the importance of this organism ..."

Prevot in 1938 (51) felt that Bergey's Manual had unjustly listed the species name necrophorus under the genus name Bacteroides which included species of no relation to each other. He, therefore, rearranged 120 species into 17 genera (6 old, and 11 new) belonging to 4 families

(2 old and 2 new) attached to two ordres (1 old and 1 new). The two Ordres were termed Bacteriales Prevot and Actinomycetales Prevot. Under the Ordre of Bacteriales were included two familles, Bacteriaceae Cohn and Ristellaceae Prevot. The Ordre Actinomycetales included the gram negative anaerobes grouped under the famille Spherophoraceae Prevot, and the gram positive anaerobes under the famille Actinomycetaceae Buchanan emend.

Bergey's Manual, seventh edition, of 1957 (11) carried a foot note expressing doubt as to the validity of the generic term of Sphaerophorus. This genus name was critized in as much as the term Sphaerophorus had been employed previously for designating a lichen. Bruce Fink (24) included this classification in his book on The Lichen Flora of the United States, the classification being arranged as follows:

Class Ascolichenes
Sub Class Gymnocarpeae
Order Caliciales
Family 13 Spherophoraceae
Genus Sphaerophorus

Sphaerophorus fragilis (L.) Persoon, 1794. Soil and Rocks, Maine and New Hampshire.
Sphaerophorus globosus (Huds.) Vainio, 1903. Soil, Rocks and Trees, Maine, New Hampshire, Washington, Oregon, and California.

Prevot (52) listed two foot notes in his manual to discount the criticism made by Bergey's Manual and to point out a reason the generic term Spherophorus should remain valid. He points out that contrary to the Botanical Nomenclature Code, the lichens are named using two different botanical terms. Because the lichens consist of a symbiotic relationship between an alga and a fungus, two separate botanical names are conjugated to describe each separately as a matter of convenience (1). Prevot

believes that the "protislologistes," as a consequence, can not claim a monopoly on the use of this generic name and, therefore, the term remains generically correct in the bacteriological nomenclature (52).

Lahelle and Thjotta (40) in 1945 proposed an entirely different genus name for the species. They suggested that the Fusobacterium genus and S. necrophorus should not be listed under different family's because they appear to be closely related, and the Bacteriodes-like grouping was misleading. Furthermore, the use of the generic terms Actinomyces and Spherophorus was also misleading. They proposed instead, the generic term of Necrobacterium Jonsen and Thjotta. This would not only point towards the main pathogenic action of the organism, the necrosis, but would also point towards Family XI, the Bacteriaceae. Listed under this Family are two genera closely related to S. necrophorus, the genus Fusobacterium and the genus Leptotrichia. Dack et al. (16, 17) objected to the use of the genus name Bacteroides because it included gram positive and gram negative species of widely divergent types. They also investigated a species the French had isolated from numerous pathological conditions in man, Bacterium funduliformis (Halle) Dack.

Another difficulty in classifying this group of organisms involves the existence of several interrelationships between S. necrophorus and other species. Dack et al. (17) compared the human pathogen, Bacterium funduliformis with the animal pathogen Bacterium necrophorum Lehmann and Neumann and found that they could be considered as constituting a single species because there was no clear-cut method for separating the two. Bergey's Manual (11) accepted the results of this study and, at present, considers the two species synonymous. However, Prevot has always

upheld the individuality of these two species and continues to list them as two separate species (4). Berens (4) tried agglutination as a means for distinguishing between the two species because there was no one other known criterion that could provide a clear cut differentiation. He found that S. necrophorus agglutinates rapidly with large agglutinins within the drop of red blood cells of a chicken and a sheep. S. funduliformis is incapable of this agglutination action. However, this is not as yet serologically definite since only four strains of the former and eleven strains of the latter species were used.

Although Bergey's Manual (11) lists Fusobacterium fusiforme Knorr as a separate genus, Prevot (52) includes it as a separate species under the genus name of Fusiformis Topley and Wilson. Berens (3) utilized the bacteriostatic action of gentian violet to demonstrate the close relationship between S. necrophorus and Fusiformis fusiformis. Lahelle and Thjotta (40) were also able to demonstrate a relationship between the two because common antigens were present, but the two were also pathogenically different. Fusobacterium caused no hemolysis of red blood cells present in an agar medium (poured into a petri dish) and no pathological conditions in laboratory animals.

Not only is there a close relationship between S. necrophorus and other gram negative anaerobes, but there is also a morphological similarity in regards to the extraordinary plasticity exhibited by it and the Nocardia revisan genus belonging to Family II Actinomycetaceae Buchanan. The genus Nocardia differs from the genus Sphaerophorus by its gram positive, aerobic, and production of true branching prop fti s. There is a uniqueness present in the genus Nocardia group concerning the

question whether they are minute fungi, higher bacteria, ancestral prototypes of both, or intermediates (23). Erickson (23) states that, "... there are innumerable instances in which nature prodigally overlaps man-made taxonomic boundaries. ...One instance is the gradual transition from one group to the other. ...there is no difference in kind, only one of degree."

In classifying the species of the genus Sphaerophorus; Bergey's Manual (11) uses two main groups, the non-motile species and the motile species (S. bullosus Breed). Under the former grouping, the seventeen remaining species are divided into gas producers and non-gas producers, which are further divided into two groups depending upon their requirement for serum and ascitic fluid. The slow liquifaction of gelatin by several of the species is a further differentiating criterion. Nine species were isolated only from man, four were isolated from the intestinal canals of mammals, two species were isolated from warm blooded animals including man, one species was isolated from domestic fowl, and one from guinea pigs. Several Americans, French, and English workers along with a German, a Japanese, and a Russian were credited with isolating and defining the species belonging to the genus Sphaerophorus.

Prevot (52) lists the motile organisms under the genera Sphaerocillus and Fusocillus. He further lists the Spherophorus species under two main groups according to their gas producing ability. The serum or ascitic fluid requirement further divides the species into four groups while the slow liquifaction of gelatin serves to distinguish further one of the species from the rest. Prevot includes two additional species, S. funduliformis (considered synonymous to S. necrophorus by Bergey's Manual) and

S. pseudonecrophorus Harris and Brown which has not been included by Bergey's Manual.

The usual descriptive characteristics based upon morphology, biochemical reactions, pathogenic capacity, and origin have been inconclusive for the identification of S. necrophorus. In recent years there has been a search for new and more novel methods of identification for the organism.

Gentian violet was first used by Glanetz and Rettger (54) for the isolation of fusiform bacteria. Beerens (3) later used the bacteriostatic effect of gentian violet to differentiate between gram negative anaerobes such as Ristella, Spherophorus and Fusiformis. The results indicated that the uninhibited Spherophoraceae group was different from the genus Bacteriodes and that Spherophorus and Fusiformis appear to be closely related genera.

In 1942 Prevot suggested that the type of fermentation be used for the identification of the anaerobic bacteria (31). Guillaume et al. (31) studied the volatile fatty acids of C-1 to C-6 produced by 215 strains of anaerobic bacteria (55 sporulating and 160 non-sporulating) distributed among 43 different species. Because of the precision of the chromatographic paper method and the consistancy of the results obtained, this appeared to be an important species specific characteristic for the identification of the anaerobic bacteria. Guillaume (32) et al. later investigated the process of the formation of the volatile fatty acids by using the Spherophorus freundi (Freund) Prevot strain 165 for the degradation of L(+) serine and L(-) threonine. The results from this study as compared to those obtained by other authors indicated that the processes

of decomposition are frequent among the gram negative anaerobic bacteria. This strain quantitatively transformed L(+) serine into acetic and butyric acids, ammonia, carbon dioxide and hydrogen. Pyruvic acid appeared during the reaction before it was transformed into acetic acid. L(-) threonine is decomposed into propionic acid, ammonia and carbon dioxide with cetobutyric acid making a brief appearance. Beerens et al. (5) point out another species specific property besides the fermentation type of volatile acids, the degradation of threonine into propionic acid. This is not only species specific but also allows for distinguishing between four groups of bacteria belonging to the Spherophorus, Fusiformis and Bistella convexa genera. The homogenicity of the genera Bistella convexa, Spherophorus and Fusiformis was also confirmed.

Hemagglutination appears to be another method for classifying the group. Beerens (4) used hemagglutination to differentiate between Spherophorus necrophorus and Spherophorus funduliformis. Tardieux (61) believes that the power to hemagglutinate is a distinctive characteristic of S. necrophorus as the human strains do not possess this power. Lahelle and Thjotta (40) used hemagglutination, agglutination reactions, and absorption tests (to verify the serological tests) to demonstrate the similarity between S. necrophorus and S. funduliformis. Beveridge (9) showed two well defined and separate groups by cross-agglutination tests on twelve strains of Bacillus necrophorus Flugge isolated from bovine livers and jaw disease in kangaroos and wallabies. Law (41) found the, "...S. necrophorus antigens, derived principally from bovine sources, are very homogenous in nature." He further states that, "... the complement affects a broader, more complete union between antigen and antiserum,

than can be demonstrated by the use of antigenic agglutinins."

Cooked meat medium has been used in the past for cultivating the organism. Hagan (33) reported that unknown substances present in meat act as a catalase on the hydrogen peroxide formation by the organism and because of this effect cooked meat media will support good growth of the organism. Orcutt advised the addition of serum to ordinary laboratory medium. Cunningham, according to Grant (29), used ascitic fluid for optimum growth. Lahelle and Thjotta (40), and Slanetz et al. (54) grew Fusobacterium on meat medium enriched with potato extract, which was used to replace serum, with brilliant green present to inhibit contaminants. Lahelle and Thjotta also used ascitic fluid in growing their Necrobacterium necrophorus Jonsen and Thjotta isolant. Blood agar (rabbit, sheep, human) has also been used (29, 15, 40). The use of serum bouillon or serum agar bouillon by Orcutt was later replaced with more complex media. Grant (29) reports that Shaw grew the organism on a medium consisting of veal infusion, peptone, dextrose, and cystine. Beveridge (9) employed cooked heart or brain medium and found that a cooked brain and liver medium was able to maintain the organism's viability from two to five weeks. West et al. (66) maintained stock cultures on an egg-meat-brain medium and used a glucose, cysteine, tryptone, beef extract medium for regular transfers. Beerens et al. (5) and Guillaume (31) have cultivated the organism on veal infusion medium containing one percent glucose, and on a tryptic peptone, meat and yeast extract plus glucose medium. Lahelle and Thjotta (40) on the other hand, demonstrated growth in only two percent peptone when the organism was grown in an atmosphere made anaerobic by the burning of hydrogen gas. Tunnicliff (63) modified Haslam's liver-brain

medium, which also includes gelatin, buffer salts, and ferric ammonium citrate, and reported uninterrupted viability of nine strains for 460 days of storage at room temperature. Thomas (62) added prekeratin extract digest of sheep hoof horn to a nutrient agar medium to grow Fusifromis nodosus after a veal infusion, horse serum, cystein medium failed. For the most part, growth is of short duration in these media (except for Tunnicliffs liver-brain medium), if growth will occur, and frequent transfers of the organism into fresh media must be made (29, 34). Cooked meat media frequently supported better growth of contaminating organisms beside supporting uncertain growth of S. necrophorus according to Grant (29). When he employed Difco fluid thioglycollate medium (22), he was able to obtain better results.

> In the early isolation attempts, pus cultures were not retained for subsequent isolation attempts because S. necrophorus was rapidly overgrown by contaminating bacteria from the pus. When it was discovered that the organism could compete favorably with the contaminants when cultured in fluid thioglycollate medium, the pus cultures were retained through several transfers making repeated isolation attempts possible. Stock cultures of the organisms were assigned strain notations and carried in the thioglycollate medium.

Because cooked meat media gave uncertain growth and growth characteristics that were not common for the organism, Grant (29) attempted a study of growth factors with the development of a chemically defined or "synthetic medium" as the long range objective. Grant found that not only do contaminating organisms grow better than S. necrophorus in the cooked meat medium but that growth will vary; while one worker can utilize the medium successfully, another worker is unsuccessful. Moreover, there are unknown substances such as growth factors and other nutrients present in varying

quantities. It must pointed out that until these unknown growth factors are resolved and a synthetic medium developed, future classification of the organism will remain difficult.

According to Grant, not only did the thioglycollate medium allow S. necrophorus to compete favorably with contaminants, but the medium could also be used as a basal medium for establishing a synthetic medium. Casitone, sodium chloride, glucose, and cystine were found to be essential for growth. However, both casitone and yeast extract components of the thioglycollate medium contain nutrients which are not defined as to quantity, or chemical structure. Another draw back is the frequency in which transfers must be made to fresh medium. Grant transferred his cultures every twenty four hours while using a forty eight hour growth for inoculation purposes (29). Canada (12) and Law (42) transferred every three to four days. These workers experienced frequent or intermittent difficulty in maintaining vigorous growth in thioglycollate. Law (42) felt that virulence was affected by the repeated transfers through the thioglycollate medium. Grant (29), Canada (12), and Law (42) experienced trouble with transfer failures after one to two months cultivation.

West and Lewis (66) were perhaps the first to have studied and reported on the growth of non-sporulating anaerobic bacteria (29 strains) from the intestinal tract in a chemically defined medium. Their stock cultures were maintained on an egg, brain medium. A synthetic medium was utilized in the study of sixteen growth promoting substances. This synthetic medium consisted of seventeen amino acids (found to be contaminated), trace mineral salts, glucose, sodium lactate and glycerol.

The results obtained were erratic and could not be confirmed; apparently their strain of S. necrophorus failed to grow in this medium. The overall purpose was to find the unknown growth factors present in a tryptone, glucose, cysteine medium.

Grant (29) later used this synthetic medium with purified amino acids as a basal medium for his study on the effect of various growth factors. However, this medium also failed to support growth for him; therefore, another basal medium was used employing a vitamin free enzymatic hydrolysate of caesin, the same mineral solution previously used, five tenths of a percent of glucose, and cysteine. Although the first basal medium failed to support growth in the presence of fourteen growth accessories and liver extract, the second basal medium supported growth of three strains in the presence of twelve growth accessories. Grant believed that the reason for growth in the initial and the serial transfers was that growth accessories were carried over in the unwashed cells. When he washed the cells twice with sterile buffered saline, only one strain of the three used grew in the synthetic medium and yet all three of the washed strains grew well in the thioglycollate medium. This one strain that grew in the initial transfer failed to grow in a subsequent transfer.

Because of the poor results obtained upon the addition of growth accessories, Grant attempted an analytical approach using yeast extract, glucose, sodium chloride, and l-cystine as the basal medium. This approach involved fractionating casitone. The vitamin free acid hydrolysate of caesin with tryptophan added apparently could not replace casitone. On the other hand, a fat insoluble, charcoal adsorbed, ether-extract (almost strepogenin-like) eluate, which was heat stable, could replace casitone

and supported the growth of six strains of S. necrophorus.

Guillaume et al. (31) were able to cultivate S. freundi strain 165 on a synthetic medium composed of seven mineral salts, glucose, glycerol, sodium acetate, glycine, twelve amino acids, and vitamins of the B-complex. Addition of threonine to this medium produced propionic acid in a quantity proportionate to the amount of amino acid added.

PROCEDURES, RESULTS AND DISCUSSION

Isolation Attempts, Method, Isolants, and Stock Cultures

On three separate occasions, fresh bovine liver, lung, reticulum and diaphragm abscesses were obtained through the cooperation of John Morrell and Company of Sioux Falls, South Dakota and the Federal Meat Inspectors present at the packing plant. Abscessed portions of the infected organs were removed by an employee of John Morrell and Company or by one of the inspectors. These dissected portions of the organs, containing the abscessed areas, were placed into plastic bags and stored in an insulated cooler containing ice.

From these bovine abscesses (from one to five centimeters in diameter), forty six attempts to isolate Sphaerophorus necrophorus were made. Of these forty six attempts, 30 percent failed to show growth, 20 percent showed contaminating growth present, and 50 percent gave characteristic isolants showing good growth and apparently an absence of contaminating organisms. Several isolants came from abscesses found on the reticulum of several steers where a nail or a piece of wire had penetrated the reticulum wall. One abscess was found on the diaphragm of a steer near a piece of wire which had penetrated the reticulum wall. Isolants taken from abscessed lungs were similar to the liver abscess isolants. The Federal Meat Inspectors had noted several instances where an abscess grew into the gall bladder, which contained great amounts of a pus-like material that had a fetid odor similar to the foul odor characteristic of the liver abscesses.

Isolants were also obtained from other animals. Two isolants were

cultivated from cholera-like abscesses of one centimeter diameter found on a chicken liver. These two isolants changed into filamentous forms from thick, gram negative rods after three weeks cultivation in a fluid thioglycollate medium. However; thick, square-ended, gram positive rods appeared in large numbers after two more weeks of cultivation. Four isolants were obtained from abscessed lungs of a deer which had died as a result of complications developing from a case of foot rot.

The seven isolants used for the amino acid studies included: S7, obtained from a three centimeter diameter abscess on the junction of the reticulum and liver; A, I, and C, from small (less than two centimeters in diameter) liver abscesses; and S2, from a three centimeter liver-rumen abscess; and V, from a large (four centimeters in diameter) liver abscess. The isolant "O" was one of Law's (41) bovine liver abscess isolants.

Isolant S7 was used almost exclusively throughout the amino acid investigation. This isolant possessed long gram negative filaments after a number of passages through a fluid thioglycollate medium. When this isolant was inoculated into the amino acid medium, long granulated or bi-polar staining rods were formed. A characteristic fetid odor, or acid-like foul odor, and large amounts of gas were produced by the isolant. Cultural characteristics in the amino acid medium was of a silky, evenly dispersed type of growth which settled to the bottom of the tube as a hard pellet upon ageing of the culture. Although the organism gave vigorous growth for two and one half months after isolation, the growth vigor began to diminish to a point where further attempts to transfer the isolant failed. A new culture of this isolant was then taken out of

a liver-brain storage medium. This liver-brain isolant began to show poor growth within two to three weeks. Washing the cells twice with a phosphate buffered physiological saline solution appeared to bring renewed growth vigor to the organism. Originally the isolant was designated S7, signifying that it came from the seventh abscess of a group of abscesses obtained on a third trip to John Morrell and Company. This S7 notation was further broadened to include the information that it was grown for a while in a liver-brain medium, LS7, and that it was washed twice, LS7W2.

Each abscessed area was first swabbed with an alcoholic iodine solution. A one cubic centimeter syringe with an 18-gauge needle and a one tenth cubic centimeter amount of a fluid thioglycollate medium was inserted into the abscess. A small amount of pus-like or necrotic material was drawn into the syringe. This material was inoculated into fifteen milliliters of Difco fluid thioglycollate medium (22). Growth generally occured within twelve hours at 37°C.

No attempt was made towards ensuring purification of the isolants. However, nutrient agar slants and fluid thioglycollate semi-solid agar medium failed to demonstrate the presence of contaminating growth for the isolants used in the amino acid studies. Moreover, numerous smears and occasional transfer failures indicated that the cultures were not contaminated by other organisms.

Two methods for storing the isolants were used. The first method utilized Tunnicliff's modification of Haslam's liver-brain medium (63), and the second involved storing the abscesses in a deep freeze.

Growth in the liver-brain medium was initiated with a one milliliter inoculum from a thioglycollate culture. Within twenty four to forty eight

hours of incubation at 37°C, growth of the isolant was indicated by the production of gas and the changing of the blackish appearance of the liver broth to a tannish color. This growth could be seen as a flocculent material in the liver broth portion of the medium. The liver-brain stock culture tubes were placed into a large coffee can, which was covered with aluminum foil, and stored at room temperature. The vaseline-mineral oil plug at the liver broth surface will harden at room temperature, thereby preventing aeration and dehydration by means of an air-tight seal. When it was desired to sub-culture from the liver-brain medium, the latter was placed in the incubator overnight and one milliliter of the fluid portion was transferred to thioglycollate the next morning. Growth occurred within twelve hours. Unless the cells are washed, they may survive transferring for only one week to four weeks in the fluid thioglycollate medium. Isolants have initiated growth in the thioglycollate medium from the liver-brain medium after five to six months of storage. For the most part, the vigor of the organism seems to have been increased, although the number of possible transfers or passages through the thioglycollate medium may have been decreased. The isolants appear, after several thioglycollate transfers, to possess the same cultural, growth, and morphological characteristics as they had when first inoculated into this medium. Moreover, one can transfer out of the same liver-brain medium culture tube many times over this period of five months and still obtain good growth each time.

Storage of the abscesses, which were not used for immediate inoculation of the thioglycollate medium, in the deep freeze did not appear to prevent growth of S. necrophorus even after eleven months of storage. To

initiate growth from these frozen abscesses, they were thawed out at a room or a 37°C temperature overnight. An inoculum taken from any one of the abscesses showed that gas was being actively produced. Smears from the inoculum and the initial thioglycollate culture contained an abundance of gram negative filaments. Although growth and morphological characteristics may appear typical of F. necrophorus for several days, contaminating organisms usually took over within two days. One isolant from a large, five centimeters in diameter, liver abscess gave an apparently pure culture of the organism. The exceedingly long filaments from this abscess grew for about a month and then failed to grow when transferred. Nine other isolation attempts from frozen abscesses showed the presence of contaminating organisms. The micro-flora of these abscesses appeared to have as the predominating organisms gram positive staphylococcus cells, gram negative diplococcus cells, and large gram positive coccoidal cells occuring in packets of four cells.

Another method for storing the organisms at a low temperature has also been tried. Culture tubes containing ten milliliters of a fluid thioglycollate medium were inoculated with two tenths milliliters of an inoculum of the organism and were then stored at 20°C. This inoculum did not change in size or amount until the tubes were incubated overnight at 37°C. Within a twelve hour period there was abundant growth of the organism in the culture medium. It has been possible to store the isolants at this temperature without loss of viability for fourteen days.

The maintenance of active cultures for the immediate inoculation of the thioglycollate base test media involved regular transfers of the isolants through fresh thioglycollate medium. These transfer periods

occurred every two days if broth (without any agar) was used and every four days if agar (0.075%) was used. This was to insure the continued growth of the isolant. Occasionally, one transfer may fail to initiate growth; this would, therefore, require further transfer attempts. In most cases growth can be initiated from transfers taken from fluid thioglycollate (0.075% agar) cultures up to fourteen days old. Twenty four hour cultures were used for inoculating the test media. This early growth (between twelve and twenty four hours) generally represented the most vigorous growth phase of the organism. To ensure rapid and positive growth, the immediate stock culture tubes were inoculated with 1.0 milliliter of inoculum. An inoculum of 0.2 or 0.3 milliliters could also be used, but growth in fifteen milliliters of fluid thioglycollate medium is slower and not as sure with the smaller inoculum.

Morphology

Smears made directly from the abscess and smears made from the thioglycollate cultures, after a long period of growth and transfers, not only exhibit difficulty in staining with the Grams method but also when a simple stain using congo red or safarin is used. Crystal violet or methylene blue works well but it is difficult to distinguish the bacterial cell clearly as to form or from debri particles which also take up the dye.

Ziel-Nielsen carbol-fuchsin stain has worked the best. It can be used with the Gram method if a coating of water is left on the slide and the full concentration of the dye (one milliliter amount) is placed into the water. The isolant cells appear to be enlarged by the dye and finer structures within the cells show up clearly such as polar bodies,

granules, and vacuoles. Generally, these structures within the cell give the appearance of a beaded form to the isolant cell. Diluting the dye with a layer of water on the slide can produce the effect of staining the cell body a light pink while staining the granules present inside the cell a dark blue. The main disadvantage for using the full concentration of dye is that it will also stain-up debri such as agar particles or chemical secretions of the cells. However, at times a full concentration of the dye must be used if the cells are to be seen at all. This is especially true for smears made directly from the abscesses where the presence of pus cells and debri make it difficult to distinguish the organism.

All sizes of filaments from exceedingly long (three or four microscope fields) to short, rods (long to very short), and cocci (small to very small) have been observed. Generally, as the number of transfers increases, rods become predominating and these rods in turn will further break down to coccus groups. Under the influence of an environmental change, rods or longer filaments are again formed from the coccus groups or the bacillus forms. Even when conditions remain the same, cultures where the bacillus forms have predominated may start producing great amounts of gas and change into long filaments. Penicillin will cause the coccoidal forms or the bacillus forms to produce filaments. The filaments may, many times, be found to be entangled with each other or may appear to be breaking up into the bacillus forms. Coccus groups sometimes appear as short streptococci groups or may appear singly (egg-shaped cells, occasionally with a banded appearance) or may appear in clusters. The thickness of the cells may vary from very thin to swollen.

Granules and vacuoles have been observed in the filaments and rods. Polar bodies and swollen spore-like bodies have also been observed. A few spheroids appeared when sodium chloride was left out of the medium. Isolant cells on recent isolation are generally even-staining with the use of any aniline dye. Both Canada (12) and Law (41) observed the presence of swellings or spheroids on the filaments present in the abscess material; however, this has not been the case for the isolants mentioned in this paper.

Washed Cells

Although Dack (15) and other investigators have referred to S. necrophorus as being a strict or obligate anaerobe, both Grant (29) and Beerens (5) have been able to wash the cells by means of a centrifuge and buffered physiological saline without any apparent loss in the viability of the organism. Grant washed the strains that he used to avoid carry-over of growth factors with the culture medium inoculum.

Because a large inoculum, two tenths milliliters, was used to inoculate the test medium, an attempt was made to wash the isolant cells and compare their growth in the test medium with the growth of the unwashed cells.

The cells were spun down twice and washed twice with a sterile phosphate-buffered physiological saline without any apparent loss in growth vigor. This was tried at room temperature and at 10°C using a refrigerated centrifuge. Rapid growth within twenty four hours occurred from two tenths milliliters inoculum in ten milliliters of the thioglycollate medium. Growth could also be obtained from the buffered saline

suspension of the isolant cells even after forty eight hours. Three isolants growing well unwashed showed equally good growth after washing. Moreover, the washing appears to stimulate the viability of an organism showing poor growth. Two isolants that were showing poor growth, grew vigorously and rapidly after being washed. The unwashed cells failed to grow upon being transferred whereas growth of the washed cells continued to increase in viability. On the other hand, five isolants showing very poor growth grew out well on thioglycollate broth after washing while the unwashed cells failed to grow when transferred. However, subsequent transfers of these washed cells also failed to show signs of growth.

Washed cells were not used as an inoculum in the study of the amino acids. There did not appear to be any significant difference in the growth of washed and unwashed cells in the test medium, provided that both were in a vigorous state of growth, to justify the expense of time involved in washing the cells. Moreover, the amount of utilizable nitrogen in the form of amino acids carried-over would be small and not as critical as would be the carry-over of growth factors in Grants study (29).

The use of unwashed cells was also continued because a large amount of cells seemed necessary in order to initiate growth, regardless whether washed or unwashed cells were used. Such a large number of cells would also carry-over available nitrogen and amino acids even when washed cells were used because of the presence of these nitrogenous compounds within the isolant cell body.

Materials and Techniques

Two sizes of culture tubes were used. For maintaining a volume

of medium adequate for the amount of inoculum placed into the test media, 2 centimeters by 17 centimeters pyrex glass tubes of fifty milliliters total volume were filled with fifteen milliliters of Difco fluid thioglycollate medium. The test media culture tubes were all of uniform diameter, thirty cubic centimeters total volume, clear pyrex glass, 1.6 centimeters by 15 centimeters; and could be used as colorimeter tubes.

Growth of an isolant in a culture medium suspension was measured by the amount of light that could be transmitted by the suspension. The amount of light that is transmitted is inversely proportional to the concentration of the suspension. In order that a linear measure of growth could be made, the percent of light transmittance was read as optical density. This optical density reading is inversely proportional to the percent of light transmittance, and can be calculated by subtracting the logrithmic value for the percent of light transmittance from the logrithm of 100, or $2 - \log t$ = optical density. Optical density units represent a product of the isolant cell size and number, and were read on a Coleman photoelectric colorimeter using a 590 Mu filter (2, 13). At least three readings were taken; before, during and after maximum growth. These three or more readings were made within an eighteen to thirty-two hour growth period to insure obtaining a reading representing the maximum stationary phase of growth by the organism in the medium. The measure of growth in the various test media, therefore, represents the optical density of the culture, calculated from percent light transmittance, read for the maximum stationary phase of growth. This stationary phase of growth represents the total or maximum growth of the organism resulting from the addition of a nutrient to the incomplete basal thioglycollate

medium. By measuring the maximum growth of the isolant as a result of the nutrient added, definite growth responses between graded amounts of nutrients or between different nutrients could be compared and evaluated.

Each test medium was made up in duplicate tubes. The first tube of the duplicate set was made up for twenty milliliters of the test medium. A ten milliliter pipette was used for thoroughly mixing the constituents of the medium and for transferring a ten milliliter portion to the second tube. Each set of tubes were individually checked for the pH of the medium and adjusted to a neutral pH when necessary. A pH range of 6.8 to 7.0, using "pH hydrion" paper and N/1 NaOH or N/1 HCl, was closely maintained to ensure growth and to prevent caramalizing the glucose upon heating. These tubes were autoclaved for a ten minute period at fifteen pounds per square inch pressure. The autoclave was brought down within a ten minute period, the tubes were quickly removed and cooled in cold water. After the tubes had cooled, they were immediately inoculated with one of the isolants.

Grant (29) used either a loopful, 0.05 or 0.10 milliliters of cells as an inoculum. According to his thesis, the results were more variable using a small inoculum than the results obtained using larger inocula. In the present study the inoculum consisted of a 0.2 milliliters suspension of cells obtained from the immediate stock culture medium of fluid thioglycollate which was well mixed by stirring with a pipette. This large inoculum ensured good growth and yet showed an optical density reading of less than 0.01 even though the inoculum could be seen as a light, silky or cloudy suspension. Because only a final reading of growth was made, the size of the inoculum did not appear to interfere

with obtaining a representative index of growth resulting from a certain nutrient. Moreover, growth would fail if an essential nutrient was left out; thereby, indicating perhaps that the medium carried over with the cells, or the nutrients present within the cells, had little influence on stimulating growth.

Optical density readings varied between duplicate tubes by only 0.01 to 0.03 for the majority of the readings taken. An experiment where quadruplet sets of tubes were used also showed optical density readings of similar precision.

Basal Medium

Before a chemically defined medium, containing only the basic nutritional requirements, can be determined; an incomplete medium must be devised to study the replacement of a complex nutrient substance. This incomplete medium is defined as the basal medium which has one essential component, such as the complex nutrient to be studied, left out and will support little or no growth by itself. The purpose of this study was to replace the caesin-peptone casitone with simpler substances, preferably amino acids. Although Grant (29) had made an attempt to fractionate casitone and thereby isolate a growth factor or factors, the present study was directed towards replacing casitone entirely with amino acids. This was done in order that later studies need concern themselves with only the vitamins and other growth factors present in yeast extract or casitone (13, 57).

The factors influencing a good basal medium involve the nitrogen and carbon source essential for cell structure, a utilizable energy source,

mineral balance, vitamins and other growth accessories, and a buffer system. In the course of this study, the employment of a basal medium yielding very little if any growth when the nitrogen source was left out was found to be advantageous.

Grant (29) developed a basal medium from Difco fluid thioglycollate medium. The complete composition of this medium is listed in Table I, page 42. When Grant fractionated this medium by the elimination of each component in turn, and determined growth under an aerobic and an anaerobic environment; he found that casitone, glucose, sodium chloride, and l-cystine were essential for growth. The thioglycollic acid was important only in establishing anaerobic conditions when physical means such as anaerobic jars were not employed. The agar further aided in maintaining anaerobioses by slowing the diffusion of air into the medium. Resazurin served merely as an oxidation-reduction potential indicator. Canada (12) further substantiated the essential nature of these constituents listed by Grant. Each of the constituents of fluid thioglycollate medium are described in the Difco Manual (22).

The casitone portion consists of peptones resulting from the pancreatic-enzyme digestion of caesin. This caesin-peptone possesses a high tryptophan content and a good buffering capacity.

According to a special report on the Constituents of Bacteriological Culture Media by the Society for General Microbiology (60), forty five percent of the weight of yeast extract consists of amino acids. The nitrogen fractions found in autolysed brewers yeast extract were calculated to 16 percent nitrogen. These fractions were described in percent amounts as total nitrogen, 11.2; amino nitrogen, 3.9; ammonia nitrogen,

TABLE I. COMPOSITION OF DIFCO FLUID THIOGLYCOLLATE MEDIUM

Components		Amount per liter
Casitone	15	grams
Yeast extract	5	
Glucose	5	
Sodium chloride	2.5	
l-Cystine	0.5	
Thioglycollic acid	0.3	milliliter
or		
Sodium thioglycollate	0.5	gram
Agar	0.75	
Resazurin	0.001	
Distilled water	1000	milliliters

0.08; and the purine base nitrogen, 0.62. Moreover, yeast extract contains growth accessories which serve to stimulate bacterial growth. Among these growth accessories, required in minute amounts, are found the naturally occurring vitamins of the B-complex.

Bacto-dextrose serves as the energy source. This monosaccharide is a, "...specially prepared d-glucose of unusual purity" (22). Other sugars as well as starch, proteins, alcohol and heavy metals, are absent from this preparation. The usual amount employed in liquid media is a 0.5 percent concentration.

The amino acid l-cystine or l-cysteine-HCl has been reported by Grant (29) and Valley (65) to play a role in an oxidation-reduction system. Grant believed that it could also have other functions since no growth occurred in its absence even under anaerobic conditions.

The sodium chloride employed is of a reagent grade.

The Difco Manual (22) describes sodium thioglycollate as a, "...product used in culture medium for testing the sterility of biological and other materials containing heavy metal compound preservatives, such as the mercurials." Toxicity of the metallic preservatives is annuled by the active sulfhydral group which also acts to lower the oxidation-reduction potential of the medium. On the other hand, this product is described in the Difco Manual as being toxic for some organisms, especially with inocula containing very few cells.

In the course of the present study, each constituent of fluid thioglycollate was studied and evaluated for its effect on the growth of S. necrophorus.

Agar (0.075 percent) was found to serve several functions. One

function was to decrease the rate of air or oxygen diffusion into the medium. The second function was to distribute the isolant cells over a wider area of the medium, and apparently to lengthen the maximum stationary growth phase. On the other hand, resazurin had very little affect on the amount of growth. This indicator dye served only to indicate the oxidation-reduction potential of the medium. Although it appeared to have some influence on the appearance of the growth of an isolant, a concentration of 1:100 failed to have any detrimental effect on growth.

A study of the casitone portion involved four methods. The first method supplemented casitone with each of the fluid thioglycollate constituents separately. Figure 1, page 45, is a bar-graph comparing the optical density readings of media containing vitamin free casamino acids (plus other constituents of the thioglycollate basal medium), casitone only, casitone and yeast extract only, casitone and glucose only, casitone and cystine only, and casitone and cysteine hydrochloride only. Sodium thioglycollate was the only other basal thioglycollate medium constituent present in the last five media mentioned above. The medium consisting solely of casitone and glucose produced growth equalling one half of that produced by the vitamin free casamino acids-thioglycollate medium. Casitone supplemented with yeast extract produced growth approximately one fourth of the maximum growth. Cystine and cysteine-hydrochloride did not appear to cause any increase in growth over that produced by casitone. However, later experiments indicated that cystine does serve as a stimulatory growth nutrient when vitamin free casamino acids is used.

Replacement of casitone with other nitrogen sources was the second method used for the study of the casitone requirement. Upon the careful

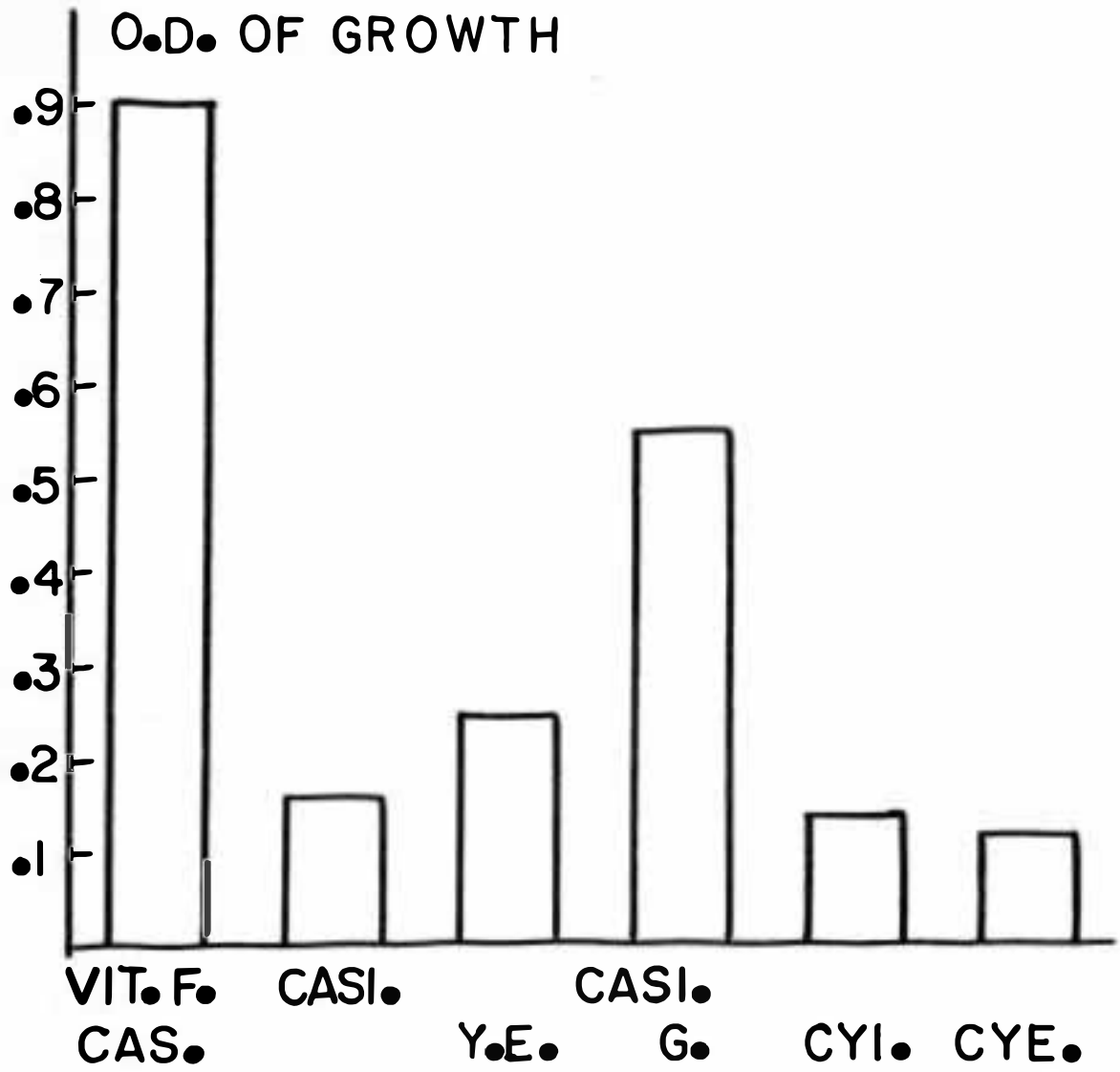

Figure 1. Bar Graph Showing Casitone Being Supplemented with Each of the Other Basal Thioglycollate Constituents

O.D., optical density; VIT. F. CAS., vitamin free casamino acids basal thioglycollate medium; CASI., casitone; Y.E., yeast extract; G., glucose; CYI., cystine; CYE., cysteine-HCl.

adjustment of pH, casitone can be replaced by caesin, vitamin free caesin, beef peptone, a casitone dialysate, casamino acids, vitamin free casamino acids, and the eighteen amino acids found in caesin. Bactopeptone failed to support growth of the organism. While Prevot (5) states that peptone water gives poor growth, Lahelle and Thjotta (40) have found good growth under an anaerobic atmosphere. Glutamic acid, alanine, aspartic acid and arginine were also incapable of supporting growth of the organism either individually or as a group.

Figure 2, page 47, shows a graph of the growth response when graded amounts of casitone were used. In the absence of casitone, only slight growth was supported by the medium. Growth is apparently directly proportional to the amount of casitone present in the medium until the concentration of casitone reaches one percent. At this concentration, the growth tapers off into a plateau which continues through the addition of one and one-half percent of casitone to the medium.

The fourth step in the casitone study involved dialyzing casitone. A cellophane bag was made from a strip of cellophane tubing twenty-four centimeters wide and thirty centimeters in length. Forty milliliters of a six percent concentration of casitone was placed into this cellophane bag which was then placed into a beaker containing two hundred milliliters of distilled water. After the casitone had dialyzed for seventy-two hours in a refrigerator, the two hundred milliliters of the casitone dialysate was added to the other thioglycollate medium constituents. This casitone dialysate had acquired an oily, silky consistency of a tannish hue. Three isolants (A, O, V) gave abundant growth within twenty-four hours after being inoculated into this dialysate medium. Isolant V gave abundant

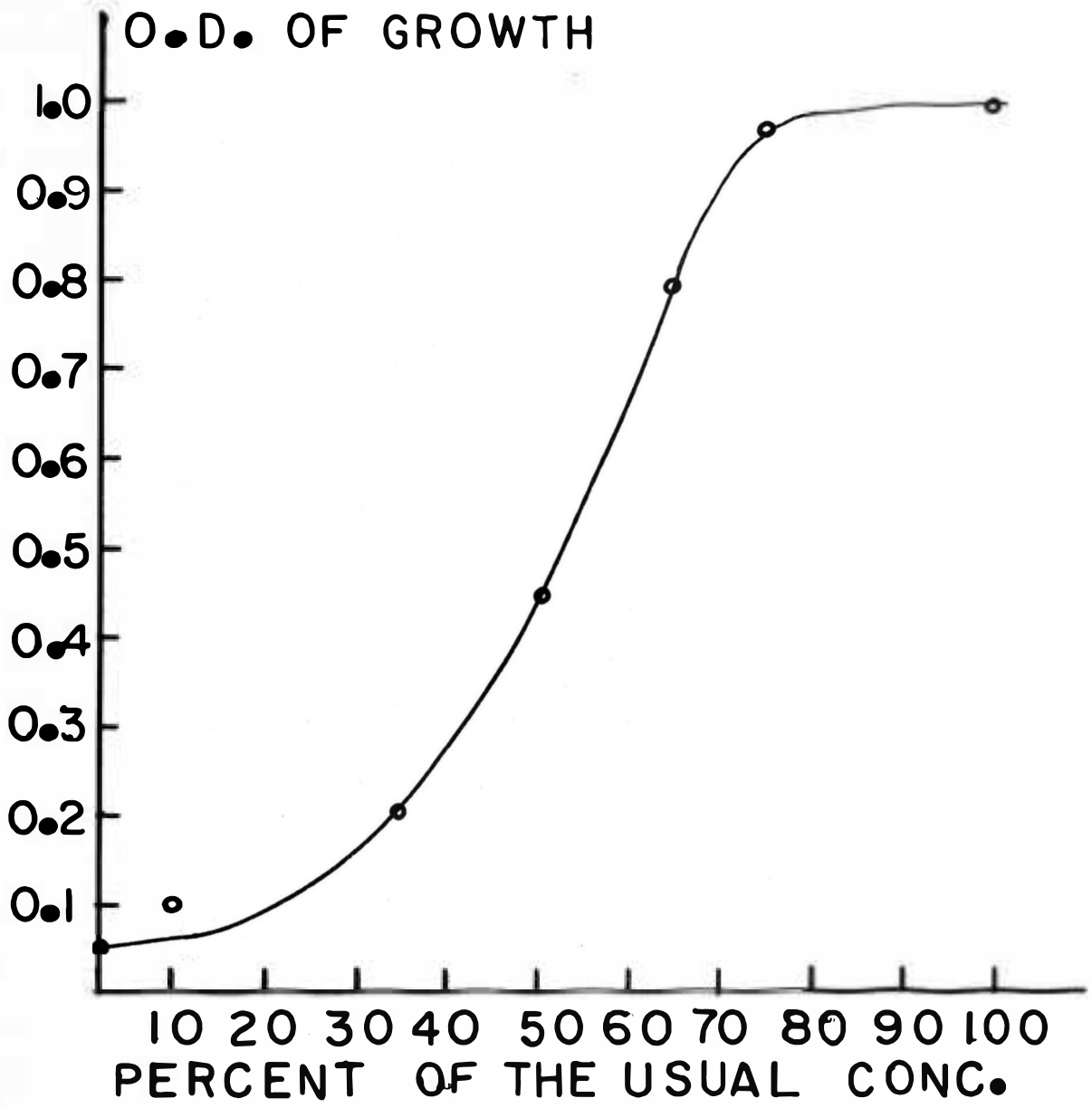

Figure 2. Casitone Growth Response Curve

The usual concentration of casitone is 15 grams per liter of medium.

growth through four serial transfers in this medium. This dialysate was heat stable as well as being stable in the presence of strong acid (pH 2) and strong alkali (pH 13). McBain (43) states that:

> A sample of commercial cellophane soaked in water only, instead of in 64% ZnCl solution, was but partially permeable to ordinary molecules. ...where the proportion retained is parallel with the number of C atoms in the molecule. It may therefore be used to study molecular size. Cellophane that has not been swollen is practically gas tight.

McBain further mentioned that the use of cellophane membranes cannot be extended above the smallest colloid ranges although they do possess the advantage of being chemically inert and insensitive. The pore size appears to be around four millimicrons. This ability to hold back molecules the size of sucrose indicates that the amino acids present in casitone and not the peptides are responsible for growth.

The ability of the organism to grow in a medium containing Bacto-Casamino Acids or Bacto-Vitamin Free Casamino Acids further substantiated the view that only the amino acids and not the peptides or peptide linkages were necessary for growth. Bacto-Casamino Acids are a product of the acid hydrolysis of casein. According to the Difco Manual (22), it can be used for nutritional studies, microbiological assays, and the preparation of synthetic or chemically defined media. It has been recommended in culture media where amino acid mixtures are required for the nitrogen source. Total nitrogen of the casamino acids equals ten percent while the sodium chloride content is fourteen percent and the ash content is twenty percent. On the other hand, Bacto-Vitamin Free Casamino Acids is similar in that it is an acid hydrolyzed casein product but differs in that the vitamins have been removed. This mixture of essentially amino

acids has been recommended for use in microbiological assay media and in studies on the growth requirements of micro-organisms. The total nitrogen content for vitamin free casamino acids is seven percent, the sodium chloride content is thirty-eight percent and the ash content is forty-one percent.

The replacement of casitone with vitamin free casamino acids showed that the organism required the amino acid components of casitone with little apparent need for peptides or peptide linkages. However, the effect of each component of the basal medium on growth still remained to be determined.

The effect on growth of graded amounts of vitamin free casamino acids was the first study conducted in the direction of a quantitative growth response. As figures 3 and 4, page 50, and Table II, page 51 indicate, there is a definite growth response. The utilization of 1.0 percent glucose in the basal medium rather than the usual 0.5 percent, gave a more reproducible response curve. Moreover, the amount of growth for 1.0 percent glucose appeared to be slightly above that for the 0.5 percent concentration. Mannitol could not be substituted for glucose in these media. This substitution was attempted in an effort to eliminate the problem of caramalization of the reducing sugar glucose. Sucrose also proved to be of no value.

The use of graded amounts of yeast extract demonstrated that it also had a decided stimulatory influence on growth. Apparently there is a definite need for vitamins or other growth accessories. When casitone is used as the nitrogen source, yeast extract is not required, thus strengthening the concept that casitone contains a necessary growth acces-

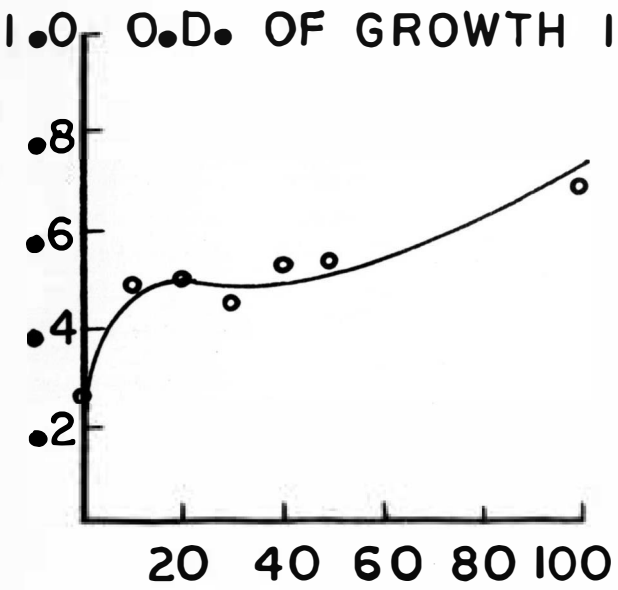

Figure 3. Vitamin Free Casamino Acids with 0.5% Glucose

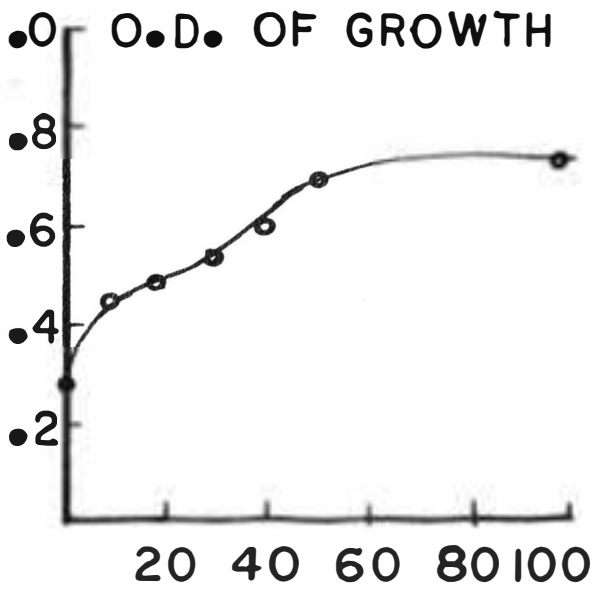

Figure 4. Vitamin Free Casamino Acids with 1.0% Glucose

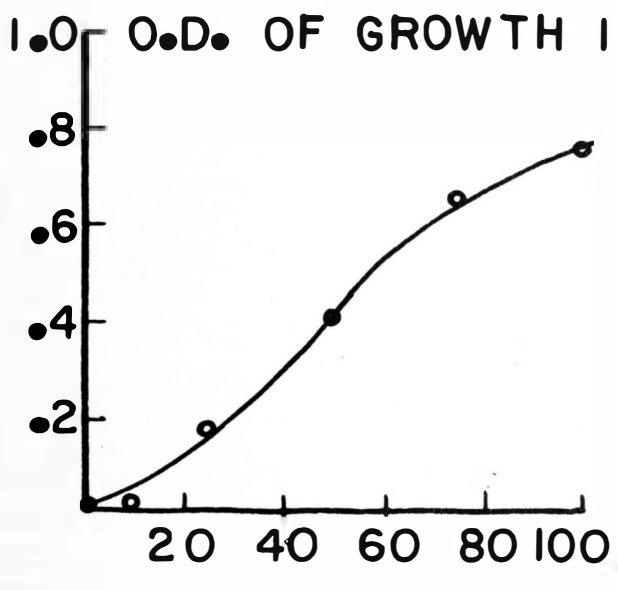

Figure 5. Yeast Extract with 0.5% Glucose

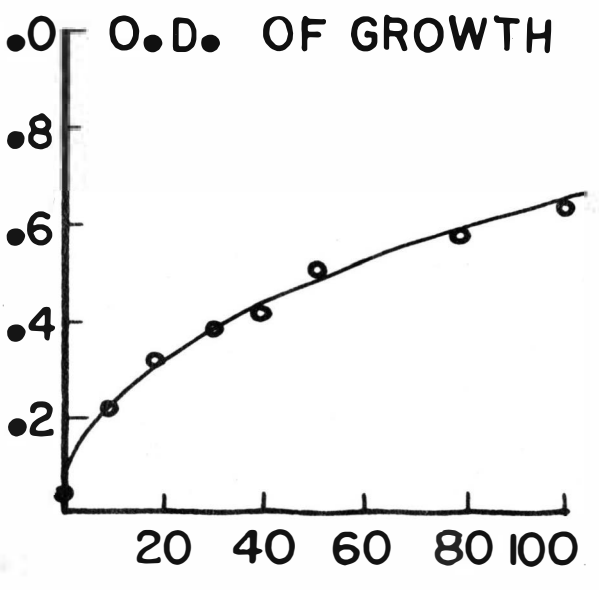

Figure 6. Vitamin Free Casamino Acids with .25% Yeast Extract 1% Glucose

TABLE II. GROWTH RESPONSE OF THE LS7 ISOLANT IN GRADED AMOUNTS OF VITAMIN FREE CASAMINO ACIDS AND YEAST EXTRACT

Concentration	Ave. O.D.			
	Yeast Extract	Vit. Free Casamino	Vit. Free Casamino	Vit. Free Casamino
Grams Per 100 ml Medium	0.5% glucose	0.5% glucose	1% glucose	0.25% y.e. 1% glucose
1.5		0.68	0.72	0.62
1.0	0.76			0.59
0.75		0.54	0.69	0.50
0.60		0.54	0.60	0.40
0.50	0.76			
0.45		0.42	0.54	0.38
0.38	0.66			
0.30		0.52	0.50	0.34
0.25	0.42			
0.20				
0.15		0.51	0.45	0.23
0.12	0.18			
0.10				
0.05	0.02			
0.00	0.01	0.27	0.29	0.07

Ave. O.D. -- optical density readings representing the average maximum growth for a set of duplicate tubes.

Optical density was determined by a Coleman photoelectric colorimeter.

Concentration -- amount of nutrient measured in grams added to the incomplete thioglycollate basal medium.

0.25% y.e. -- represents one half of the amount of yeast extract present in the fluid thioglycollate medium.

1% glucose -- twice the usual amount found in the thioglycollate medium.

sory or accessories. Table II, page 51 and figure 5, page 50 not only show that growth does not occur when yeast extract is left out, but they also show that (up to a limit) growth is almost directly proportional to the amount of yeast extract present in the medium.

Because one half of the usual concentration of yeast extract produced one half of the amount of maximum growth, a third growth study was attempted. This growth response experiment involved using graded amounts of vitamin free casamino acids, one percent glucose, and one half of the usual five tenths percent concentration of yeast extract along with the other components such as sodium chloride, cystine, and sodium thioglycollate. Using this combination, a response to yeast extract was obtained as seen in Table II and figure 6. This was more in line with the requirements for a basal medium to be employed in the amino acid study. Growth without the nitrogen source was so small that it would not adversely affect the evaluation of the amino acid growth responses. This was in sharp contrast to a basal medium employing the usual one half of a percent concentration of yeast extract. Here growth could occur in the absence of any other nitrogen source which could definitively influence the amino acid growth study results.

A sodium chloride tolerance level growth response study was attempted after the observation of the unusual morphological changes that could be induced by leaving this salt out of the medium. Growth is also affected by this deletion. Generally, growth on the initial transfer is slow but within forty-eight or seventy-two hours the growth vigor increases sharply and large amounts of gas are given off to form a froth-like layer at the surface of the medium. Through several transfers

adverse morphological characteristics of the cells are evident. Not only do the cells swell up and become greatly enlarged, but filament length and production increases. Several cases were noticed where spherical swellings known as spheroids developed on the long filaments. As shown in Table III, page 54 the isolant used (LS7W2) could not adapt to a medium that did not contain any sodium chloride. It grew best when the sodium chloride concentration was increased from the thioglycollate amount of 0.25 percent to a concentration of 0.8 percent. A concentration of 1.5 percent appears to give the same amount of growth as does the 0.25 percent concentration. However, growth diminishes rapidly beyond the 1.5 percent concentration. Although growth of significance occurs in a 2.5 percent concentration, there is no significant growth at a sodium chloride concentration of 4.0 percent.

Cystine actually plays a minor role in the basal medium. For the study of growth responses to the amino acids, cystine is left out of the basal medium. However, not only did cystine prove to be the most important amino acid required for growth, but it also appeared to exert a stimulatory effect on the other amino acids, vitamin free casamino acids and the incomplete thioglycollate basal medium as demonstrated in Table IV, page 55.

Table V, page 56 lists the micrograms of cellular nitrogen per milliliter of a ten milliliter culture sample present in the cells of the LS7 isolant grown in vitamin free casamino acids media. The micrograms of total precipitable cellular nitrogen per milliliter of culture medium was determined by means of a semi-micro Kjeldahl method (68). Calculation of the preciptable cellular nitrogen was accomplished according to

TABLE III. SODIUM CHLORIDE TOLERANCE LEVEL SHOWN BY THE LS7W2 ISOLANT

Percent NaCl Concentration	Average Optical Density	Hours
0.0	0.09	13
0.1	0.36	30
0.25	0.51	
0.5	0.66	
0.8	0.73	
1.0	0.71	
1.5	0.50	35½
2.0	0.35	
2.5	0.14	
4.0	0.01	
5.0	0.00	

Number of hours incubation at 37°C until the isolant showed its maximum growth in the basal medium.

TABLE IV. GROWTH STIMULATION OF THE S7 ISOLANT BY THE PRESENCE OF CYSTINE IN VARIOUS MEDIA

Basal Medium	Optical Density of Duplicate Tubes			
	Without Cystine		With Cystine	
0.15% VF casa	0.03	0.03	0.30	0.30
1.5% VF casa	0.64	0.67	0.75	0.74
18 amino acids	0.64	0.62	0.77	0.75
5 amino acids	0.40	0.39	0.40	--
7 amino acids	0.44	0.44	0.55	--
Incomplete medium 1	0.01	0.02	0.09	0.11
Incomplete medium 2	0.18	0.16	0.45	0.43

Incomplete medium 1 has one half of the thioglycollate medium amount of yeast extract or .25 gm/100 ml

Incomplete medium 2 has the regular amount of yeast extract or 0.5 gm/100 ml

TABLE V. MICRO-GRAMS OF CELL NITROGEN PRESENT IN CELLS OF THE L57 ISOLANT

Basal Medium	Ave. O.D. Isolant Growth 590 Mu Filter	O.D. Isolant Cell Nitrogen 470 Mu Filter	Amount of Cell Nitrogen per ml. of culture ugm N/ml
1.5% V.F. casamino	0.79	0.59	134.9
0.75% V.F. casamino	0.41	0.42	96.0
0.0% V.F. casamino	0.26	0.15	34.5
1.5% V.F. casamino 2	0.79	0.54	125.5
1.5% V.F. casamino 3	0.65	0.39	89.0
Standard		0.45	20.6

Cell nitrogen determined by a semi-micro Kjeldahl method.

1.5% Vitamin free casamino acids basal thioglycollate medium 2 had one percent glucose instead of the thioglycollate medium amount of 0.5%.
medium 3 had 1% glucose plus one half of the usual 0.5% thioglycollate medium amount of yeast extract.

the following formula,

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times \text{concentration of standard} \times 5 \text{ fold dilution} = \text{concen. of unknown}$$

In preparing the isolant cells for the precipitable cellular nitrogen determination, the isolant cells were spun down twice into a pellet by means of a centrifuge and washed twice with a buffered saline solution. After the second washing they were washed into the kjeldahl flasks and digested by means of a sulfuric acid digestion mixture and heat.

The importance of a nitrogen source in the basal medium is further shown in Table V by the effect that vitamin free casamino acids has on the growth and nitrogen content of isolant LS7. When the vitamin free casamino acids concentration in the basal medium is reduced by one half, the optical density values measuring cell growth is also reduced by one half and the nitrogen content in the isolant cells is reduced by one third. The absence of vitamin free casamino acids from the basal medium results in a further drop in optical density to one third of the optical density of growth obtained with a complete medium and a further drop in cell nitrogen to one fourth of the cell nitrogen in a complete basal medium. The addition of twice the thioglycollate medium concentration of glucose gave an optical density reading equal to that for the regular thioglycollate medium amount of glucose. However, the cell nitrogen content for the increased concentration of glucose was slightly less than that shown by the regular thioglycollate amount of glucose. Reducing yeast extract in the basal medium by one half and increasing the glucose concentration to one percent decreased the optical density reading for cell growth by one fifth and the cell nitrogen content by one third.

These results suggest that vitamin free casamino acids is supplying nitrogen needed for growth by the cells while the yeast extract is supplying the needed vitamins and other growth factors also required for good growth by the organism. Although one percent glucose appears to have an effect on the nitrogen content of the cells, this effect is too small to be considered of significance.

The last constituent to be evaluated in the basal medium was the buffering system. Casitone is an excellent buffer in the fluid thioglycollate medium and gives a 7.2 pH to this medium. Vitamin free casamino acids have very little effect on the 6.3 pH of the basal medium other than to lower the pH to 6.0. Previous observations demonstrated that the isolant can lower the pH from 7.2 to 6.5 within twenty-four hours and further lower the pH to 5.5 after four days of incubation at 37°C. It became apparent that an adequate buffering system must be employed if the amino acid growth results were to be consistant. The isolant can initiate growth in a medium with a pH of 6.0 to 6.3, but this only rarely occurs. A pH in the range of 7.0 to 7.2 or 7.4 allows rapid growth by the organism. In order that growth will continue after it has started at a pH of 7.0, any sudden change in the pH as a result of the organisms metabolic processes is compensated for by the use of a buffering system. A phosphate buffer system utilizing di-sodium hydrogen phosphate and potassium di-hydrogen phosphate was tried and was found to work well for the conditions described. Both of the components of this buffer system were made-up in one liter amounts and stored separately in a refrigerator. These two buffer stock solutions contained 9.5 grams of anhydrous di-sodium hydrogen phosphate and 9.078 grams of potassium di-hydrogen phosphate for

a 1/15 molar concentration of each per liter of distilled water. The addition of a 6.5 volume of di-sodium hydrogen phosphate to a 3.5 volume of potassium di-hydrogen phosphate resulted in a solution having a neutral pH. When the thioglycollate basal medium constituents were dissolved with this buffer solution the resulting pH proved to be too alkaline. A ratio of a 4.0 volume of di-sodium hydrogen phosphate to a 6.0 volume of potassium di-hydrogen phosphate resulted in a more desirable value that was close to a neutral pH for the basal medium.

Amino Acids

Eighteen amino acids reported to be present in the protein caesin were used in this study according to their average percent proportions for each amino acid as reported by five sources (30, 14, 38, 27, 58). A list of the eighteen amino acids and their relative percent amounts as found in caesin and the amounts used in this study is given in Table VI, pages 60 and 61. Also listed are the molecular weights of each amino acid and the percent of total nitrogen present in each amino acid. The quantity of total nitrogen added to a ten milliliter basal medium to give the concentration of each amino acid comparable to that found in casein is also given.

A stock solution of each amino acid was made up so that one half of a milliliter volume contained a concentration of the amino acid equivalent to the amino acid's concentration in caesin. This one half of a milliliter amount was intended for ten milliliters of an incomplete basal thioglycollate medium. However, a mistake was made that resulted in a two fold dilution of each amino acid. In combining the incomplete basal

TABLE VI. RELATIVE AMOUNTS OF THE EIGHTEEN AMINO ACIDS ADDED TO THE BASAL MEDIUM

Amino Acid	Approx. Percent in Caesin	Grams Added to 10 ml Basal Medium	Mole. Weight in Grams	Percent Total Nitrogen Per Mole.	Grams Total N Per 10 ml Medium
dl-Alanine	4	0.006	89	16	0.00096
l-Arginine-Mono-HCl	4	0.006	174	32	0.00192
l-Aspartic Acid	7	0.010	133	12	0.00120
l-Cystine	0.4	0.006	240	12	0.00072
dl-Glutamic Acid	23	0.035	147	10	0.00350
l-Glycine	1	0.002	75	19	0.00038
l-Histidine	3	0.005	155	27	0.00135
dl-Isoleucine	7	0.010	131	11	0.00110
l-Leucine	10	0.015	131	11	0.00165
l-Lysine-Mono-HCl	8	0.012	146	19	0.00228
l-Methionine	3	0.005	149	9	0.00045
l-Phenylalanine	5	0.008	165	9	0.00072
l-Proline	10	0.015	115	12	0.00180
l-Serine	7	0.010	105	12	0.00120
dl-Threonine	4	0.006	119	12	0.00072
l-Tryptophan	1	0.002	204	14	0.00028
l-Tyrosine	6	0.009	181	8	0.00072
dl-Valine	8	0.012	117	12	0.00144
Total	111.4	0.179			0.02239

Continued on next page.

Percent of each amino acid as found in casein was taken from the average of five estimates (30, 14, 38, 27, 58).

The approximate percent of each amino acid was multiplied by 15 grams/liter in order to replace casitone.

Cystine was figured as 4% instead of 0.4% because of its importance in the growth of the organism.

medium with the amino acids and/or supplementary compounds, and the buffer solution; the first tube of each duplicate set was made-up for twenty milliliters of medium. Ten milliliters of the test medium, after being thoroughly mixed, was transferred to the second tube when all of the constituents had been added to the medium. Consequently, the amino acid concentration intended for ten milliliters of the test medium ended, instead, in twenty milliliters of test medium. Because the isolants grew well in the culture medium containing the eighteen amino acids in one half of their intended concentration, no attempt was made to correct the final amino acid concentration.

The amino acids were made-up as stock solutions when it was impractical to weigh them into the basal medium. A few of the amino acids will dissolve readily in distilled water at a pH of 6.0, but most of the amino acids require a pH of 7.0 or 8.0, while others require a pH in the range of 9.5 and above for the concentration of amino acid to be used. Glutamic acid is made up to one half of the total volume required with water adjusted to a 9.5 pH and N/1 NaOH is added until this amino acid goes into solution. Isoleucine will stay in solution and will not crystallize out at a refrigeration temperature if a pH of 10.0 or more is used for maintaining solution. Tyrosine will crystallize out unless it is kept near boiling temperature even when its pH is at 11. Cystine must

have a pH of 9.5 if it is to dissolve, but at a pH of 10.5 or 11 it should not be heated by itself as it will decompose. It is advisable on the first attempt to dissolve the amino acid with one half of the total volume of water desired (previously adjusted or not adjusted to a high pH value), and then adjust with N/1 NaOH until the amino acid goes into solution. The quantity of water added is below the required volume so that the final pH adjustment may give the desired value. The addition of water will lower the pH and the amino acid may be precipitated out of solution. The pH level and refrigeration served to protect the amino acids from contaminating growth for a period up to two weeks, even after numerous occasions when the stock solutions were warmed to room temperature for transferring to the culture medium tubes. Constituents are added individually to each tube along with a set amount of the incomplete basal medium and enough buffer to bring the final volume to twenty milliliters. The amino acid solutions are made up so that a five tenths milliliter volume of each is required per set of duplicate tubes. The first tube of the duplicate set is well mixed using a ten milliliter pipette, and a ten milliliter portion is then transferred to a second tube of the duplicate set. Control tubes containing the incomplete basal medium and vitamin free casamino acids were run with each experiment to determine the least and the greatest growth.

Amino Acids Supplemented with Other Nitrogen Compounds

Because the amino acids used could also be supplying nitrogen rather than being required only for their chemical structures, the concentration of the amino acids was cut back to one fifth of the normal

concentration and a compound supplying readily available nitrogen was used to supplement the amino acids. This permitted a more definite determination of growth response based on the chemical structures of the amino acids and not on the amount of available nitrogen which varies with each amino acid. Glycine, di-ammonium hydrogen phosphate and ammonium acetate were the nitrogen supplementing compounds tested. As shown in Table VII, page 64, glycine appeared to be toxic or antagonistic in the concentration required for supplementing the eighteen amino acids. Di-ammonium hydrogen phosphate produced growth equal to the growth produced by the eighteen amino acids (or vitamin free casamino acids) when used to supplement the eighteen amino acids reduced to one fifth of their regular concentration. By itself in the basal medium, using the thioglycollate medium content of yeast extract, it produced only one sixth as much growth as the eighteen amino acids when the growth on the incomplete basal medium was subtracted. The basal medium that was used with this ammonium compound was later found to be insufficiently buffered. When a phosphate buffering system was placed into the basal medium, it became very difficult to prevent the glucose from carmalizing upon autoclaving when this ammonium salt was also present in the medium. Therefore, ammonium acetate was used as a nitrogen source allowing the medium to be sterilized without carmalization. The acid nature of the acetate ion apparently compensated for the alkalinity of the ammonium ion. Moreover, the already high concentration of phosphate ions introduced into the medium by the buffer system was not further increased. The ability of these ammonium compounds to supplement either the eighteen or the four "essential" amino acids (cystine, histidine, alanine, arginine) supports the concept that

TABLE VII. THE ABILITY OF AMMONIUM COMPOUNDS TO SUPPLEMENT THE AMINO ACIDS IN GROWING THE S7 ISOLANT

Ammonium Compound	Amino Acid Medium	Average Optical Density				
		Amino Acids Reg. Amts.	Amino Acids 1/5 Amts.	Amino Acids + NH_4	NH_4 Compd.	Incomplete Basal Medium
Glycine	18 aa.	0.72	0.36	0.13	0.13	0.15
$(NH_4)_2HPO_4$	18 aa.	0.72	0.36	0.61	0.25	0.15
	4 aa.	0.45	0.25	0.45	0.25	0.15
$NH_4C_2H_3O_2$	18 aa.	0.60	0.40	0.61	0.15	0.20
	4 aa.	0.41	0.35	0.53	0.15	0.20
	5 aa.	0.40	0.18	0.38	0.24	0.08
	9 aa.	0.39	0.20	0.40	0.24	0.08

The amino acids have been cut back by four fifths of their full concentration in the basal medium.

The first five incomplete basal medium optical density readings represent growth on two different occasions. This medium contains a full thioglycollate medium amount of yeast extract and glucose.

The last two incomplete medium readings represent growth on one occasion where the yeast extract was cut back by one half and the glucose was increased to one percent.

the isolant cells are using the amino acids for available nitrogen in addition to requiring the molecular structure of the amino acids.

The amount of the ammonium compound to be weighed into solution was calculated so that one half of a milliliter portion from a fifty milliliter stock solution would supply the necessary quantity of available nitrogen. This quantity of nitrogen was used to supplement fully the amino acids present in one fifth of their regular concentration. The amount of nitrogen in the amino acid medium was thereby brought to full strength with the ammonium salt being tested.

The use of these ammonium salts was limited to the study on their ability to supplement the amino acids. The individual growth response exhibited by each amino acid appeared significant enough for the purpose of this study without further investigation.

Amino Acids Study

Addition of the eighteen individual amino acids found in caesin, in the amounts found therein, into the basal medium began the study. After the pH was adjusted to 7.0 and the medium sterilized and inoculated, growth occurred that equaled the growth on vitamin free casamino acids which, in turn, equaled the growth on casitone. Three isolants (A, I, S2) were able to grow well through two serial transfers.

In an attempt to determine which of the eighteen amino acids had a greater influence on growth, one amino acid was left out of each set of duplicate tubes in a series of nineteen pairs of culture tubes (57). The nineteenth pair were control tubes that contained all eighteen amino acids. A definite growth response was seen, dividing the amino acids into

three groups according to the duplicate tubes optical density readings. Table VIII, page 67 lists the three optical density ranges resulting from the omission of each amino acid listed from the medium.

The four "essential" amino acids (cystine, arginine, alanine and histidine) were felt to be important enough that an attempt should be made to study the growth responses for each amino acid. However, the results proved erratic. Usually the growth resulting from use of the four amino acids was only one half of that for the eighteen amino acids, but occasionally equal growth would occur. On the other hand, growth sometimes failed to start or fell below the usual expected result and equaled growth on the incomplete medium. Growth on the incomplete medium was also unpredictable, at times the growth was quite low and at other times the growth was excessively high.

The failure to secure growth, the uncertain growth occurring in the medium containing four amino acids, and the unpredictable growth occurring in the incomplete basal medium made necessary further modifications of the basal medium. These modifications have been mentioned previously under the basal medium section. They involved the incorporation of a buffer system, decreasing the yeast extract to one half of its usual concentration and an increase in glucose from one half to one percent.

This modified basal medium served to maintain growth at a more consistant level. There were no sudden increases in growth from one experiment to another. The amino acids demonstrated almost the same growth response from one determination to the next. Moreover, growth was at a level that did not appreciably interfere with the other growth responses.

TABLE VIII. THREE GROUPS OF AMINO ACIDS ACCORDING TO THEIR EFFECT ON GROWTH WHEN EACH BY ITSELF IS LEFT OUT OF THE AMINO ACID MIXTURE

O.D. 40-50 Range	O.D. 60-65 Range	O.D. 70-80 Range
Essential Amino Acids	Growth Stimulatory Amino Acids	Non-Essential Amino Acids
Cystine	Lysine	Valine
Arginine	Leucine	Aspartic Acid
Alanine	Phenylalanine	Methionine
Histidine	Tyrosine	Serine
	Glycine	Threonine
	Tryptophan	Glutamic acid
	Proline	
	Isoleucine	

These optical density ranges represent the amount of growth supported by an amino acid basal medium when only one of the eighteen amino acids has been left out of the medium.

The amino acids are listed according to the greatest effect on growth shown by the deletion of each amino acid by itself from the amino acid mixture.

The groups of the amino acids are named according to Snell (57) for their individual effect on growth when one amino acid of the group is left out of the medium.

Table IX, page 69 summarizes the individual effect on growth by each of the four amino acids that were thought to be important. Apparently cystine and histidine are important, but arginine and alanine seem to exert only a small influence on growth. On the other hand, cystine appears to account for the total growth present in the medium containing the four amino acids because of its individual effect on growth of the organism. Histidine was still considered important since it supported growth almost as well as cystine.

Both cystine and histidine were considered the most important amino acid for use in the next experiment. Each of the eighteen amino acids, beginning with histidine, were added to cystine one by one through a series of duplicate tubes until a total of sixteen amino acids was used. To these sixteen amino acids, arginine and alanine were added. Table X, page 70 gives the results of this experiment. A rather fluctuating group of optical density readings of growth show an increase over the cystine and histidine growth response with the addition of each amino acid. Glutamic acid, previously thought to be unimportant, caused a sharp increase in the total growth obtained by the S7 isolant. Alanine and arginine failed to have any stimulatory effect on this growth.

The growth supported by an individual amino acid is given in Table XI, page 71 for each of the eighteen amino acids. Ammonium acetate and fifteen hundreds of a percent of vitamin free casamino acids were used to supplement each amino acid in order that the nitrogen content would be brought towards full concentration in the test medium. The five amino acids of cystine, histidine, glutamic acid, threonine, and proline were considered to be of importance. Four amino acids consisting of tyrosine,

TABLE IX. INDIVIDUAL EFFECT OF THE FOUR "ESSENTIAL" AMINO ACIDS ON GROWTH OF THE LS7 ISOLANT

Basal Medium	O.D.	
Cystine	0.42	0.43
Histidine	0.30	0.32
Alanine	0.03	0.03
Arginine	0.11	0.07
All Four Amino Acids	0.43	0.44
0.15% Vit. Free Casamino	0.03	0.03
1.5% Vit. Free Casamino	0.75	0.74
Incomplete Basal Medium	0.02	0.00

Ten percent of the full thioglycollate basal medium concentration of vitamin free casamino acids was placed into each amino acid culture tube in order that the total nitrogen content would be brought-up closer to the basal medium nitrogen content.

TABLE X. EFFECT OF SUPPLEMENTING CYSTINE AND HISTIDINE WITH THE OTHER SIXTEEN AMINO ACIDS ON GROWTH OF THE LS7W2 ISOLANT

Amino Acid Basal Medium	Ave. O.D.
Cystine	0.29
Cystine + Histidine	0.28
2 a.a. + Lysine	0.34
3 a.a. + Leucine	0.37
4 a.a. + Phenylalanine	0.43
5 a.a. + Tyrosine	0.47
6 a.a. + Glycine	0.52
7 a.a. + Tryptophan	0.51
8 a.a. + Proline	0.46
9 a.a. + Isoleucine	0.45
10 a.a. + Valine	0.44
11 a.a. + Aspartic Acid	0.46
12 a.a. + Methionine	0.41
13 a.a. + Serine	no growth
14 a.a. + Threonine	0.49
15 a.a. + Glutamic Acid	0.68
16 a.a. + Alanine + Arginine	0.68
Vit. Free Casamino Acids	0.65
Incomplete Basal Medium	0.05

TABLE XI. INDIVIDUAL EFFECT OF EACH AMINO ACID ON THE GROWTH OF THE LS7W2 ISOLANT

Amino Acid Basal Medium	Ave. O.D.
Cystine	0.35
Histidine	0.29
Lysine	0.18
Leucine	0.20
Phenylalanine	0.25
Tyrosine	0.27
Glycine	0.22
Tryptophan	0.25
Proline	0.29
Isoleucine	0.23
Valine	0.22
Aspartic Acid	0.24
Methionine	0.21
Serine	0.26
Threonine	0.29
Glutamic Acid	0.36
Alanine	0.22
Arginine	0.19
Vit. Free Casamino Acids	0.65
$NH_4C_2H_3O_2$ + 0.15% V.F. Casamino	0.24
Incomplete Basal Medium	0.05

Ammonium acetate and ten percent of the full concentration of vitamin free casamino acids were added to each amino acid to adjust the nitrogen content.

glycine, tryptophan, and aspartic acid appeared as if they could serve to stimulate growth of the organism.

As can be seen in Table XII, page 73, the four amino acids thought to have growth stimulating properties failed to increase the growth supported by the five "important" amino acids.

Although the five "important" amino acids were able to support growth equivalent to only one half of the growth supported by vitamin free casamino acids, these five amino acids were still considered an important nitrogen source. Because these five amino acids did give good growth according to an optical density reading of 0.42, serial transfers of three isolants were made through three media containing different amino acids, and a vitamin free casamino acids medium. The purpose of this experiment was to further compare the growth supported by each group of amino acids to that supported by the vitamin free casamino acids medium. This last amino acid study involved weighing the five amino acids into one-hundred milliliters of the thioglycollate basal medium, with cystine present, for a growth comparison between it and the seven, nine, eleven amino acids and vitamin free casamino acids. From the results listed in Table XIII, page 74 the five amino acids appear to be the main amino acids required for growth. Although alanine and arginine in several instances appear to stimulate growth, the stimulation remains slight. Growth increased with each serial transfer, indicating that a period of adaptation to the amino acids is involved before their growth can equal that using vitamin free casamino acids.

Tables XIV and XV, pages 75 and 76 have been included to demonstrate that a sulfur requirement exists for the isolants used. During

TABLE XII. EFFECT ON GROWTH OF THE LS7W2 ISOLANT BY THE FIVE "IMPORTANT", FOUR "STIMULATORY", AND TWO "DOUBTFUL GROWTH STIMULATORY" AMINO ACIDS

Amino Acid Basal Medium	Ave. O.D.
Five "Important" amino acids	0.42
Four "Stimulatory" amino acids	0.19
Five amino acids + Tryosine	0.39
+ Glycine	0.40
+ Tryptophan	0.39
+ Aspartic Acid	0.39
Five amino acids + Four amino acids	0.42
Five amino acids + Arginine + Alanine	0.44
Eighteen amino acids	0.46
Vit. Free Casamino Acids	0.59
Incomplete Basal Medium	0.06

Five amino acids -- Glutamic acid, Cystine, Histidine, Proline, and Threonine.
The eighteen amino acids and V.F. casamino acids control media were slightly carmalized.

TABLE XIII. SERIAL TRANSFERS OF THREE ISOLANTS THROUGH THIOGLYCOLLATE BASAL MEDIA CONTAINING FIVE, SEVEN, NINE, ELEVEN AMINO ACIDS AND VITAMIN FREE CASAMINO ACIDS

No. of Amino Acids	Number of Serial Transfers											
	1	2		1	2	3		1	2	3	4	
	Isolant			Isolant				Isolant				
5	A	0.38	0.70	C	0.25	0.54	0.51	LS7W2	0.40	0.65	0.70	0.70
7		0.45	0.39		0.25	0.59			0.55	0.70	0.75	0.75
9		0.36	0.48		0.15	0.51	0.45		0.40	0.55	0.68	0.65
11		0.45	0.45		0.20	0.54	0.56		0.52	0.66	0.64	0.67
V.F. casa.		1.30	0.80		0.70	0.70	0.70		0.77	0.83	0.90	0.80

Serial transfers involve transferring growth from one tube into a second tube and transferring the resulting growth from this tube into another tube and so on -- .

TABLE XIV. SULFUR REQUIREMENT OF THE ISOLANT LS7W2

Basal Media	Ave. O.D.
Eighteen amino acids (-) Cystine	0.40
Eighteen amino acids (-) Methionine	0.72
Eighteen amino acids	0.81
Eighteen amino acids (-) Cystine & Methionine	0.20
Eighteen amino acids	0.76
Eighteen amino acids (-) Cystine & Methionine	0.41
Eighteen amino acids	0.68
$(NH_4)_2SO_4$ + 0.15% Vit. Free Casamino acids	0.15
Na_2SO_4 + $NH_4C_2H_3O_2$ + 0.15% Vit. Free Casamino	0.20
$NH_4C_2H_3O_2$ + 0.15% Vit. Free Casamino Acids	0.24
Incomplete Basal Medium	0.05
3 x conc. $(NH_4)_2SO_4$	0.11
3 x conc. Na_2SO_4 + $NH_4C_2H_3O_2$	0.03
Incomplete Basal Medium	0.10

0.061 grams di-ammonium sulfate or 0.084 grams di-sodium sulfate were used per tube of basal medium.

The addition of 0.015 grams of vitamin free casamino acids and/or 0.08 grams ammonium acetate served to adjust the amount of nitrogen in the medium.

The eighteen amino acids media were used as a control for growth comparison.

Methionine and Cystine were left out of the amino acid mixture on two separate occasions.

TABLE XV. THE IMPORTANCE OF CYSTINE IN COMPARISON WITH EIGHTEEN AMINO ACIDS THROUGHOUT THE COURSE OF THE AMINO ACIDS INVESTIGATION

Average Optical Density		
Cystine Basal Medium	18 amino acid Medium	Incomplete Basal Medium
0.46	0.65	0.15
0.42	0.60	0.13
0.49	0.72	0.20
0.28	0.75	0.02
0.44	0.75	0.02
0.29	0.68	0.05

0.15% vitamin free casamino acids was added to the fifth cystine basal medium.

The last three optical density readings were made on media containing one half of the thioglycollate amount of yeast extract.

the amino acids study, cystine has always had the greatest stimulatory effect on growth. If left out of the medium total growth is only one half of the potential total growth, using eighteen amino acids. Methionine, the only other sulfur containing amino acid present, apparently has little effect on growth as long as cystine is present in the culture medium. However, when both cystine and methionine are left out, growth is sharply curtailed.

Because the amino acids could not be replaced with an inorganic source of sulfur, the sulfur requirement of the isolants appears to involve organic sulfur rather than inorganic sulfur.

SUMMARY AND CONCLUSIONS

In an effort to establish a chemically defined medium for Sphaerophorus necrophorus, the casitone portion of a fluid thioglycollate derived basal medium was investigated for the nutrients required for growth. Unlike Grants (29) search for a growth factor believed to be present in casitone, the present casitone study was directed toward the replacement of casitone with amino acids found in the protein caesin.

Although seven S. necrophorus isolants were used throughout the amino acid study, one isolant in particular was used to demonstrate amino acid growth responses. This isolant came from an abscess three centimeters in diameter found on the junction of the reticulum and liver of a steer. Morphologically, it was found to be growing in long filaments upon isolation from the abscess but acquired a long bacillus form after being grown in the various amino acid basal media. Originally this isolant was designated as S7 because it came from the seventh abscess of a series of abscesses acquired on a third trip to a meat packing plant. However, transfer failures added more designating marks such as LS7, taken from storage in a liver-brain medium, and LS7W2, the liver-brain medium isolant was washed twice with sterile buffered saline.

Stock cultures of the isolants were maintained on a liver-brain medium that had previously been used for this same purpose by Tunnicliff (63). This medium maintained the viability of the isolants for as long as five months.

Washing the isolants twice with a sterile, phosphate buffered, physiological saline solution did not appear to harm these anaerobic

organisms. Growth of the washed isolants was as good if not better than the growth shown by the isolants before washing with the saline solution.

The amount of growth given by an isolant on a liquid culture medium was measured turbidometrically by the amount of light that could be transmitted through the culture medium. Optical density readings made on a photoelectric colorimeter were used in evaluating the effect on the growth of an isolant in a culture medium by the addition or deletion of a nutrient. These readings represent the maximum stationary growth occurring in duplicate culture tubes of a medium.

A basal medium for the amino acid study was derived from Grants study (29) of the Difco fluid thioglycollate medium (22). This basal medium was composed of the essential components of the thioglycollate medium as listed by Grant. These components were casitone, glucose, sodium chloride, and l-cystine. Yeast extract was added to this medium when the casitone portion was replaced with amino acids, and sodium thioglycollate was also included to establish anaerobic conditions.

The analysis of the basal medium served to establish the importance of each component. The data obtained were used to modify the basal medium. Importance of casitone for the growth of S. necrophorus was shown by its production of a definite growth response. There was almost no growth in its absence and full growth when it was present in full strength. The presence of only casitone and sodium thioglycollate in a medium supported significant growth equaling one fifth of the maximum growth. Addition of glucose to the casitone and sodium thioglycollate medium supported growth that was one half of maximum growth. Yeast extract also gave a growth response as significant as that supported by casitone when the vitamin

free casamino acids (essentially an amino acid mixture) was used to replace casitone. A sodium chloride tolerance level study and the sharper growth response obtained with twice the usual amount of glucose were other factors considered in modifying the basal medium. The removal of casitone created a problem of pH maintenance and for this reason a new buffering system was added. From these investigations, a basal medium to be employed for the study of the amino acid replacement of casitone was developed. The composition of this basal medium included 15.0 grams of vitamin free casamino acids, 2.5 grams of yeast extract, 10 grams of glucose, 2.5 grams of sodium chloride, and 0.5 gram of sodium thioglycollate per liter of a 1/15 M phosphate buffer solution. Analysis of the effect of various amino acids on growth was accomplished by replacing the vitamin free casamino acids with the amino acids to be studied.

The research study on the amino acid replacement of the caesin-peptone casitone began when not only an acid hydrolyzed caesin product was found to replace casitone, but also when a dialysate of casitone was found to support the growth of S. necrophorus. This ability to support growth of the organism by the break-down of caesin into products consisting essentially of amino acids was further substantiated by the effect on growth by the individual amino acids. A mixture of eighteen amino acids, that are present in caesin, were similarly able to support growth when the pH was adjusted to 7.0.

Following the suggestion of Snell (57), the simplest approach to determine the amino acid requirements was to omit each individual amino acid by itself from a mixture of the eighteen amino acids and then test the resulting mixtures for their ability to promote growth of the organism.

This deletion of only one amino acid from each medium resulted in the appearance of three groups of amino acids according to their ability to stimulate growth. These groups were thought to represent the "essential" amino acids, the "stimulatory" amino acids, and the "non-essential" amino acids (57). Further growth-response attempts with the eighteen amino acids and a group of four "essential" amino acids (cystine, hystidine, arginine, alanine) gave erratic results. The erratic results were blamed on the basal medium being able to support significant growth by itself that could interfere with the growth supported by the amino acids.

After modification of the basal medium only cystine and histidine appeared to give a growth response. When cystine and histidine were supplemented with the other sixteen amino acids one at a time, it was seen that three other amino acids appeared to be important. These three amino acids were glutamic acid, proline, and threonine. Four amino acids (tyrosine, glycine, tryptophan, aspartic acid), according to their individual effect on growth, appeared to have a possible growth stimulating ability. The addition, individually or as a group, of the four amino acids had no effect on the growth supported by the five "important" amino acids. Alanine and arginine also appeared as if they might have some stimulatory effect on growth but the results of this postulation were rather indefinite. Although the LS7W2 isolant grew better when these two amino acids were added, this marginal increase in growth diminished with each serial transfer. Furthermore, the other two isolants did not grow to the same magnitude as did the LS7W2 isolant when the two amino acids were present in the medium.

Serial transfers of three isolants (A, C, LS7W2) were made through

thioglycollate basal media consisting of the five "important" amino acids, the "important" amino acids plus alanine and arginine, the "important" amino acids plus the four "stimulatory" amino acids, and these eleven amino acids combined. The LS7W2 isolant apparently required a period of adaptation to the five "important" amino acids before growth in this medium would equal the growth in a vitamin free casamino acids medium.

Apparently the S. necrophorus isolants used require certain amino acids. The ability of either di-ammonium hydrogen phosphate or ammonium acetate to support good growth when supplemented with the amino acids, which have been reduced in amount by one fifth, indicates that ammonia nitrogen can be used by S. necrophorus for part of its nitrogen requirement. On the other hand, the chemical structures of certain amino acids are also necessary as indicated by the low level of growth supported by the ammonium salts and the growth response results shown by the five "important" amino acids. It is suggested that the sulfur requirement cannot be satisfied by sulfate but only by sulfur containing amino acids.

There are a number of variable factors induced by the amino acid and bacterial cell relationship that have been or have not been covered by this report. Such variables as cell membrane permeability, ammonia nitrogen availability, nitrogen carry-over in the inoculum, total number of bacterial cells used as inoculum, degree of anaerobiosis, and the sugar present certainly play a part in this study.

According to Snell (57), the three groups of amino acids which appear when one amino acid at a time is left out of a mixture fall into three groups representing those essential for growth, those stimulating growth, and

those without any apparent effect on growth. Two variables may play a part in this instance; the imbalance of amino acids (antagonistic amino acid concept) and the essential precursor role of nonessential amino acids in a restricted medium. These variables show up in the inability of the first two groups of amino acids to support growth unless one or several amino acids are added from the third group.

From the results so far obtained, there appears to be a requirement by the S. necrophorus isolants used for the amino acids cystine, glutamic acid, histidine, proline, and threonine. Because of the excellant growth obtained with the LS7M2 isolant in the medium containing five amino acids, investigations into the growth factor requirements of Sphaerophorus necrophorus appear feasible and should be undertaken.

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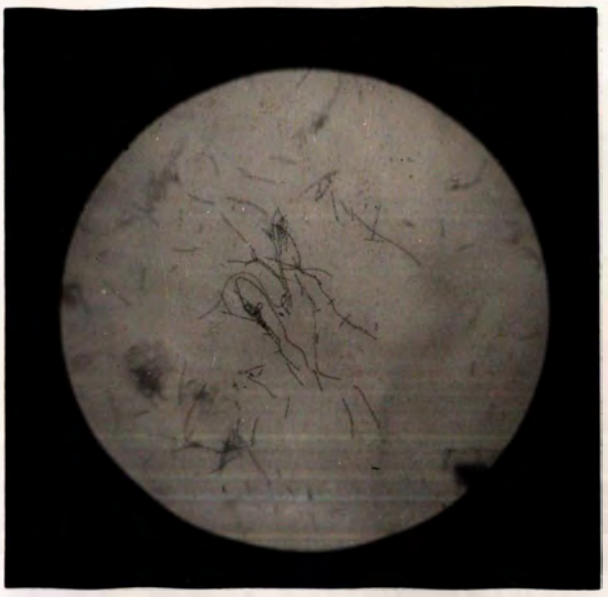
APPENDIX

Basal Thioglycollate Medium

Nitrogen source	
Casitone	15 grams
Vitamin free casamino acids 7% N = 1.5 grams	15 grams
18 amino acids 1/2 prescribed amount for casein used 8 grams = 1 gram N	8 grams
Vitamins and growth accessories	
Yeast extract 16 = 0.8 grams or 0.4 gram N	5 grams - 2.5 grams
Energy source	
Glucose	5 grams - 10 grams
Mineral	
Sodium chloride	2.5 grams
Anaerobioses	
Sodium thioglycollate	0.5 grams
l-Cystine (usually left out)	0.5 grams
Buffer system	1000 milliliters
600 ml 1/15 M KH_2PO_4 to 400 ml 1/15 M Na_2HPO_4 Store separate stock solutions in a refrigerator.	

Abscessed portions of various organs taken from beef cattle.

The long, even-staining filamentous forms of the S7 isolant after one week of cultivation on fluid thioglycollate, magnified approx. 1000 times. Stained with a full concentration of carbol-fuchsin.



S7 isolant after one week cultivation in the thioglycollate medium. Granulated filamentous forms are magnified approx. 1000 times, stained with a dilute solution of carbol fuchsin.

Bacillus and coccus forms of isolant "O". Magnified approx. 2,000 times. Stained with a full conc. of Carbol fuchsin.

Coccus stage of growth for the isolant "O". Magnified approx. 2,000 times. Stained with a full conc. of carbol fuchsin.

Granulated bacillus and filamentous forms of isolant "U". Magnified approx. 2000 times. Stained with a full conc. of carbol fuchsin.

Culture media and tubes used in study. Numbered from left to right, they are: thioglycollate culture (large size) tubes 1, 2, 3, 7, 8; a general lab. culture tube 4; a colorimeter test medium culture tube 5; liver-brain stock culture tubes 6, 7.

Granulated bacillus and filamentous forms of isolant "U". Magnified approx. 2000 times. Stained with a full conc. of carbol fuchsin.

Culture media and tubes used in study. Numbered from left to right, they are: thioglycollate culture (large size) tubes 1, 2, 3, 7, 8; a general lab. culture tube 4; a colorimeter test medium culture tube 5; liver-brain stock culture tubes 6, 7.